The human corpus luteum: functional and structural effects of maternal recognition of pregnancy.

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Declaration

I hereby declare that this thesis has been composed by myself and is my own work. Any contribution of others has been fully acknowledged. The work reported in this thesis has not been submitted in full or in part for any other degree, diploma or professional qualification.

Dr William Colin Duncan
1st July 1998

Dedication

This thesis is dedicated to my parents for their constant support and encouragement and to my friends who put up with my neglect and absences during its preparation.

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List of Abbreviations

3β-HSD	3β-Hydroxysteroid Dehydrogenase
11β-HSD	11β-Hydroxysteroid Dehydrogenase
17α-hydroxylase	17α-Hydroxylase, C17,20 Lyase
AB	Avidin-Biotin
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
bFGF	Basic Fibroblast Growth Factor
bp	Basepair
BSA	Bovine Serum Albumin
bTP-1	Bovine Trophoblastic Protein-1
c.p.m.	Counts per minute
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
COX	Cyclooxygenase
CRF	Corticotrophin Releasing Factor
CV	Coefficient of Variation
DAB	Diamino-Benzidine
DAG	Diacylglycerol
$DHKF_{2\alpha}$	13,14-Dihydro-15-keto-prostaglandin $F_{2\alpha}$
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonuclease
DTT	Dithiothreitol
eCG	Equine Chorionic Gonadotrophin
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FSH	Follicle Stimulating Hormone

G-protein	GTP binding protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GnRH	Gonadotrophin Releasing Hormone
$GnRH_{ant}$	Gonadotrophin Releasing Hormone Antagonist
H_2O_2	Hydrogen Peroxide
hCG	Human Chorionic Gonadotrophin
HDL	High Density Lipoprotein
HRP	Horseradish Peroxidase
i.m.	Intramuscular
i.v.	Intravenous
IFN	Interferon
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IgG	Immunoglobulin G
IL	Interleukin
ITP	Inositol Triphosphate
kb	Kilobase
kDa	Kilodalton
LDL	Low Density Lipoprotein
LH	Luteinising Hormone
LMP	Last Menstrual Period
MCP-1	Monocyte Chemoattractant Protein-1
MMP	Matrix Metalloproteinase
MMP-1	Matrix Metalloproteinase-1 (Interstitial Collagenase)
MMP-2	Matrix Metalloproteinase-2 (Gelatinase A)
MMP-3	Matrix Metalloproteinase-3 (Stromelysin-1)
MMP-7	Matrix Metalloproteinase-7 (Matrilysin)

MMP-9	Matrix Metalloproteinase-9 (Gelatinase B)
MMP-10	Matrix Metalloproteinase-10 (Stromelysin-2)
MMP-11	Matrix Metalloproteinase-11 (Stromelysin-3)
mRNA	Messenger Ribonucleic Acid
NBT	Nitroblue Tetrazolium Chloride
NGS	Normal Goat Serum
NO	Nitric Oxide
NRS	Non-immune Rabbit Serum
O_2•-	Superoxide Anion
OH•	Hydroxyl Radical
oTP-1	Ovine trophoblastic protein-1
$P450_{arom}$	Cytochrome P450 Aromatase
$P450_{scc}$	Cytochrome P450 Cholesterol Side Chain Cleavage
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PG	Prostaglandin
PGDH	Prostaglandin Dehydrogenase
PKC	Protein kinase C
r.p.m.	Revolutions per minute
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RNAse	Ribonuclease
RT	Reverse transcriptase
RU486	Mifepristone
s.c.	Subcutaneous
S.D.	Standard Deviation
S.E.M.	Standard Error of the Mean

SCP-2	Sterol Carrier Protein-2
SDS	Sodium Dodecyl Sulphate
StAR	Steroidogenic Acute Regulatory Protein
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween
TEA	Triethanolamine
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloproteinases
TNF	Tumour Necrosis Factor
TP-1	Trophoblastic protein-1
tRNA	Transfer Ribonucleic Acid
TSH	Thyroid stimulating hormone
u.v.	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

Publications related to this thesis

LH receptor in the human corpus luteum: lack of down-regulation during maternal recognition of pregnancy

Paper published:

Duncan WC, McNeilly AS, Fraser HM, Illingworth PJ (1996) Luteinizing hormone (LH) receptor in the human corpus luteum: lack of down-regulation during maternal recognition of pregnancy. *Hum. Reprod.* **11** 2291-2297.

Abstract published:

Duncan WC, McNeilly AS, Rodger FE, Illingworth PJ (1995) The effect of luteal rescue on LH receptor localisation in the human corpus luteum. *J. Reprod. Fertil.* (Abstract Series) **15** 40.

Induced luteolysis in the primate: rapid loss of LH receptors

Paper published:

Duncan WC, Illingworth PJ, Young FM, Fraser HM (1998) Induced luteolysis in the primate: rapid loss of luteinizing hormone receptors. *Hum. Reprod.* **13** 2532-2540.

Steroidogenic enzyme expression in human corpora lutea in the absence and presence of exogenous hCG

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The human corpus luteum continues to express the progesterone receptor during maternal recognition of pregnancy

Paper published:

Koh EAT, Illingworth PJ, **Duncan WC**, Critchley HOD (1995) Immunolocalisation of bcl-2 in human endometrium in the menstrual cycle and simulated early pregnancy. *Hum. Reprod.* **10** 1557-1562.

Abstracts published:

Duncan WC, Critchley HOD, McNeilly AS, Illingworth PJ (1994) The effect of luteal rescue on progesterone receptor localisation in the endometrium and corpus luteum. *J. Reprod. Fertil.* (Abstract Series) **13** 30.

Critchley HOD, Koh E, **Duncan WC**, Illingworth PJ (1995) Endometrial bcl-2 immunolocalisation in the menstrual cycle and pseudopregnancy. *J. Endocrinol.* (Supplement) **144** 137.

Expression of TIMP-1 in the human corpus luteum after luteal 'rescue'

Paper published:

Duncan WC, McNeilly AS, Illingworth PJ (1996) Expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the human corpus luteum after luteal rescue. *J. Endocrinol.* **148** 59-67.

Abstract published:

Duncan WC, McNeilly AS, Illingworth PJ (1995) The effect of luteal rescue on the expression of matrix metalloproteinases and their inhibitors in the human corpus luteum. *Biol. Reprod.* (Supplement) **1** 222.

Expression of TIMP-1 in the primate ovary during induced luteal regression

Paper published:

Duncan WC, Illingworth PJ, Fraser HM (1996) Expression of tissue inhibitor of metalloproteinases-1 in the primate ovary during induced luteal regression. *J. Endocrinol.* **151** 203-213.

Abstract published:

Duncan WC, Lunn SF, McNeilly AS, Fraser HM, Illingworth PJ (1996) Expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the steroidogenic tissues of the marmoset. *J. Reprod. Fertil.* (Abstract Series) **17** 97.

The effect of luteal 'rescue' on the expression and localisation of MMPs and TIMPs in the human corpus luteum

Paper published:

Duncan WC, McNeilly AS, Illingworth PJ (1998) The effect of luteal 'rescue' on the expression and localization of matrix metalloproteinases and their tissue inhibitors in the human corpus luteum. *J. Clin. Endocrinol. Metab.* **83** 2470-2478.

Abstract published:

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The human corpus luteum: reduction in macrophages during maternal recognition of pregnancy

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Rodger FE, **Duncan WC,** Largue PY, Fraser HM, Illingworth PJ (1997) Distribution of leukocytes in the human corpus luteum throughout the luteal phase and in simulated early pregnancy. *Hum. Reprod.* (Abstract Book 1) **12** R-228.

The research in this thesis has been the subject of an invited review paper and included in some symposia at scientific meetings

Invited Review:

Duncan WC (1998) The human corpus luteum: tissue remodelling during luteolysis and maternal recognition of pregnancy. *Rev. Reprod.* In preparation.

Meeting Symposia Abstracts:

Fraser HM, **Duncan WC**, Illingworth PJ, Lunn SF, Rodger FE (1997) Endocrine control of the corpus luteum. *Hum. Reprod.* (Abstract Book 1) **12** O-61.

Fraser HM, **Duncan WC**, Erickson GF, Harrison D, Illingworth PJ, Lunn SF, Rodger FE, Young FM (1997) Control of cell death in the ovary. *J. Endocrinol.* (Supplement) **152** S20.

Presentations at Scientific Meetings

Oral Presentations

Duncan WC, Critchley HOD, McNeilly AS, Illingworth PJ (1994) The effect of luteal rescue on progesterone receptor localisation in the endometrium and corpus luteum. Society for the Study of Fertility, Annual Conference, Southampton, UK, July 1994.

Duncan WC, McNeilly AS, Rodger FE, Illingworth PJ (1995) The effect of luteal rescue on LH receptor localisation in the human corpus luteum. Society for the Study of Fertility, Annual Conference, Dublin, Ireland, July 1995.

Duncan WC, McNeilly AS, Illingworth PJ (1996) Differential localisation of Matrix Metalloproteinases and their inhibitors by *in situ* hybridisation in the human corpus luteum. Society for the Study of Reproduction, 29th Annual Meeting, London, Ontario, Canada, July 1996.

Poster Presentations

Duncan WC, McNeilly AS, Illingworth PJ (1995) The effect of luteal rescue on the expression of matrix metalloproteinases and their inhibitors in the human corpus luteum. Society for the Study of Reproduction, 28th Annual Meeting, Davis, California, USA, July 1995.

Duncan WC, Lunn SF, McNeilly AS, Fraser HM, Illingworth PJ (1996) Expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the steroidogenic tissues of the marmoset. Society for the Study of Fertility, Annual Conference, Nottingham, UK, July 1996.

Duncan WC, Rodger FE, Illingworth PJ (1996) Luteal 'rescue' is associated with a reduction in macrophage numbers in the human corpus luteum. Simpson Symposium 9, Edinburgh, UK, August 1996.

Invited Presentations

Duncan WC (1995) The Corpus Luteum: An unfinished jigsaw. Edinburgh Obstetrical Society 177th Session, Edinburgh, UK, October 1995.

Duncan WC (1996) The corpus luteum and maternal recognition of pregnancy. Centre of Reproductive Biology, Edinburgh, UK, November 1996.

Duncan WC (1996) The corpus luteum: the fat controller of the menstrual cycle. Department of Physiology, University of Edinburgh, Edinburgh, UK, December 1996.

Duncan WC (1998) It's not what you do but where you do it: remodelling in the corpus luteum. Gynaecological Travellers Club, Edinburgh, UK, November 1998.

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Chapter 1

Introduction

"These yellow bodies of the ovary have been puzzling to scientists ever since they were first described by Regner de Graaf"

Corner, 1943

1.1 Overview

The corpus luteum is an endocrine gland of the ovary. It is formed from the dominant follicle after the oocyte has been ovulated and has all but disappeared at the time of menstruation. During its short life-span, it has a huge capacity for hormone synthesis and secretion. Indeed, weight for weight, it is the most active endocrine gland in the body (Zeleznik and Fairchild Benyo, 1994). The principle hormone secreted by the corpus luteum is progesterone, a C_{21} steroid. Progesterone alters the morphology and function of the endometrium. This results in its stabilisation and prepares the uterine environment for implantation of the blastocyst (Bell, 1983). The integrity of the corpus luteum is thus fundamental to the establishment of pregnancy.

The human corpus luteum has a functional life-span of approximately 14 days (Lenton and Woodward, 1988). This is known as the luteal phase of the ovarian cycle, during which the synthetic capacity of the corpus luteum changes. Progesterone production increases in the early-luteal phase to reach a maximum in the mid-luteal phase. However, in the late-luteal phase, the functional integrity of the corpus luteum is lost and progesterone production falls (Behrman *et al.*, 1993). Progesterone withdrawal destabilises the endometrium and stimulates inflammatory cascades which result in endometrial shedding (Salamonsen and Woolley, 1996). Thus, failure of luteal progesterone production is the cause of menstruation in a non-conception cycle.

The structure of the corpus luteum also changes during the luteal phase. After ovulation, the cystic follicle, with its avascular granulosa cells, is transformed into

the solid, highly vascular, corpus luteum. The mid-luteal corpus luteum measures up to two centimetres in diameter and can be clearly identified on the surface of the ovary (Zeleznik and Fairchild Benyo, 1994). It has an orangy-red colouration which reflects its high vascularity and lipid content. However, in the late-luteal phase, as progesterone production falls, the corpus luteum regresses structurally (Behrman *et al.*, 1993). At the time of menstruation, the corpus luteum can still be identified in the ovary, but it has become a small, relatively avascular, fibrous remnant (Corner, 1956). This loss of the corpus luteum from the ovary is known as luteolysis. During luteolysis, the corpus luteum, therefore, loses both its functional and structural integrity.

The functional and structural integrity of the corpus luteum are maintained in the presence of an implanting blastocyst. Human chorionic gonadotrophin (hCG) is synthesised in logarithmically increasing amounts by the trophoblast of a developing pregnancy (Lenton and Woodward, 1988). Luteolysis is prevented by exposure of the corpus luteum to hCG from the conceptus. This means that progesterone continues to be produced and the structure of the corpus luteum is maintained. The continued presence of progesterone maintains the uterine environment and prevents menstruation, allowing implantation and early embryonic development (Stouffer, 1988). Therefore, in a conception cycle, the corpus luteum is 'rescued' from luteolysis and this is fundamental for the maternal recognition of pregnancy.

The corpus luteum is therefore one of the lynchpins of human reproduction. It is the primary interface between menstruation and maternal recognition of pregnancy. An understanding of the physiological processes central to the corpus luteum would have wide implications for both fertility promotion and prevention. However, the molecular events surrounding functional and structural luteolysis, and how they are prevented by hCG during maternal recognition of pregnancy, are still not clear in women. The corpus luteum remains one of the great enigmas of modern reproductive biology.

This chapter reviews the current understanding of the control of the primate corpus luteum. It begins by discussing the ovarian cycle and gonadotrophin control of follicular growth. The molecular mechanisms of ovulation, luteinisation and formation of the corpus luteum are then discussed. This is followed by a review of our understanding of the functional properties of the corpus luteum. The next aspect to be covered is our current understanding of luteolysis, luteal

involution and the factors implicated in its control. Maternal recognition of pregnancy and luteal 'rescue' are then discussed. This highlights the adequacies and the deficiencies in our current understanding and the need to develop novel model systems to study the human corpus luteum. This chapter serves as an introduction to the following experimental chapters which report studies of the human corpus luteum during maternal recognition of pregnancy.

1.2 Folliculogenesis

Any discussion of the corpus luteum must begin with a discussion of general ovarian physiology, in particular follicular growth and development. This is because the structure, function and control of the corpus luteum all have their origin in the preovulatory follicle. In addition, it is increasingly clear that there are a great many parallels in the control of the follicle and that of the corpus luteum (Zeleznik and Fairchild Benyo, 1994; Fortune, 1994; Gougeon, 1996). Normal folliculogenesis is necessary for the development of a normal corpus luteum.

1.2.1 The Ovarian Cycle

The ovary is the endocrine gland responsible for the release of the female gamete, the oocyte. As well as growth, development and release of the oocyte, the ovary secretes the steroid hormones which are responsible for female secondary sexual characteristics and set the hormonal milieu for the establishment of pregnancy. During the follicular phase of the ovarian cycle, cystic follicles, containing oocytes, grow, mature and secrete increasing amounts of oestrogen (Adashi, 1994). Eventually one follicle is selected to become the dominant follicle and the other follicles die by the process of atresia (Gougeon, 1996). The high concentration of oestrogen secreted by the mature dominant follicle triggers a positive feedback response in the hypothalamo-pituitary axis resulting in a gonadotrophin surge (Zeleznik and Fairchild Benyo, 1994). This luteinising hormone (LH) surge results in ovulation, with follicular rupture, release of the oocyte and transformation of the follicular remnants into the corpus luteum (Behrman *et al.*, 1993). The corpus luteum secretes large amounts of progesterone during the luteal phase of the ovarian cycle (Fig. 1.1). After luteolysis, progesterone secretion fails and the ovary enters the follicular phase of the ovarian cycle once again (Adashi, 1994).

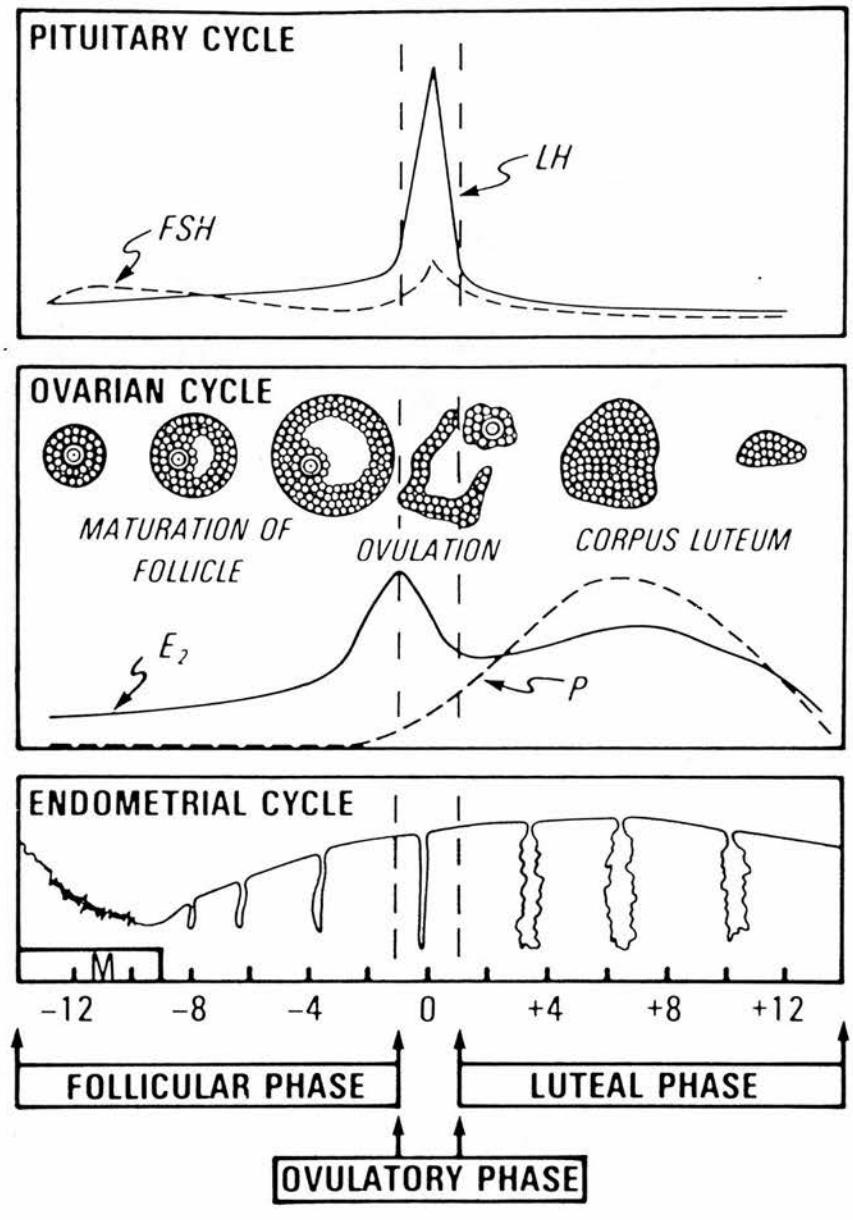

Figure 1.1

Hormone profile of the human menstrual cycle

Diagrammatic representation of the changes in serum concentrations of the main sex steroids, 17β-oestradiol (E) and progesterone (P), and the gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH), throughout the menstrual cycle. The days of the cycle, and the timing of menstruation and ovulation, are indicated. The menstrual cycle incorporates the follicular and luteal phases of the ovarian cycle.

1.2.2 Anatomy of the Ovarian Follicle

Mammalian ovaries have a pool of primordial follicles consisting of an oocyte, arrested in prophase of meiosis I, and a single layer of flattened epithelial-like granulosa cells (Zeleznik and Fairchild Benyo, 1994). During reproductive life, a cohort of these follicles begin to grow and mature. The factors responsible for the initiation of follicular development are still not clear (Fortune, 1994). However, once follicular growth begins, the follicle has one of two fates: ovulation or atresia. Only a tiny proportion of follicles ovulate (Fig. 1.2).

The primordial follicle may remain dormant in the ovary for more than forty years. The first stage in its transition to a primary follicle is characterised by enlargement and proliferation of the granulosa cells, and an increase in the size of the oocyte (Gougeon, 1996). When the follicle has acquired three to six layers of granulosa cells, some stromal cells near the basal lamina become aligned parallel to each other around the primary follicle. These fibroblast-like cells change into epithelioid-like cells, capable of steroidogenesis, and stratify into the theca cell layers. At this stage the follicles develop an independent blood supply (Gougeon, 1996). This is the preantral stage of follicular development.

The appearance of the antral cavity starts with the development of small fluid-filled spaces, within the granulosa cell layer, that coalesce to form the antrum. From this point onwards there is a specific group of granulosa cells which surround the oocyte. These granulosa cells form the cumulus cells which have cytoplasmic connections to the oocyte. There appears to be a stratification of the rest of the granulosa cells, as those nearest the basement membrane become more columnar in shape (Gougeon, 1996). At this stage, in the human, the follicles measure 180 to 250 μm in diameter. Through the accumulation of fluid, in the antral cavity, and the proliferation of granulosa and thecal cell layers, the follicles continue to grow until they measure between two and five millimetres in diameter. This process takes about 85 days and these small antral follicles can be found at all stages of the ovarian cycle (Zeleznik and Fairchild Benyo, 1994). It is not clear what factors are involved in controlling this stage of follicular growth. However, the next stages of follicular growth are gonadotrophin-dependent (Fig. 1.2).

The size of the follicle destined for ovulation increases greatly during the follicular phase of the ovarian cycle by cellular multiplication and accumulation of follicular fluid. This process requires trophic stimulation by follicle stimulating

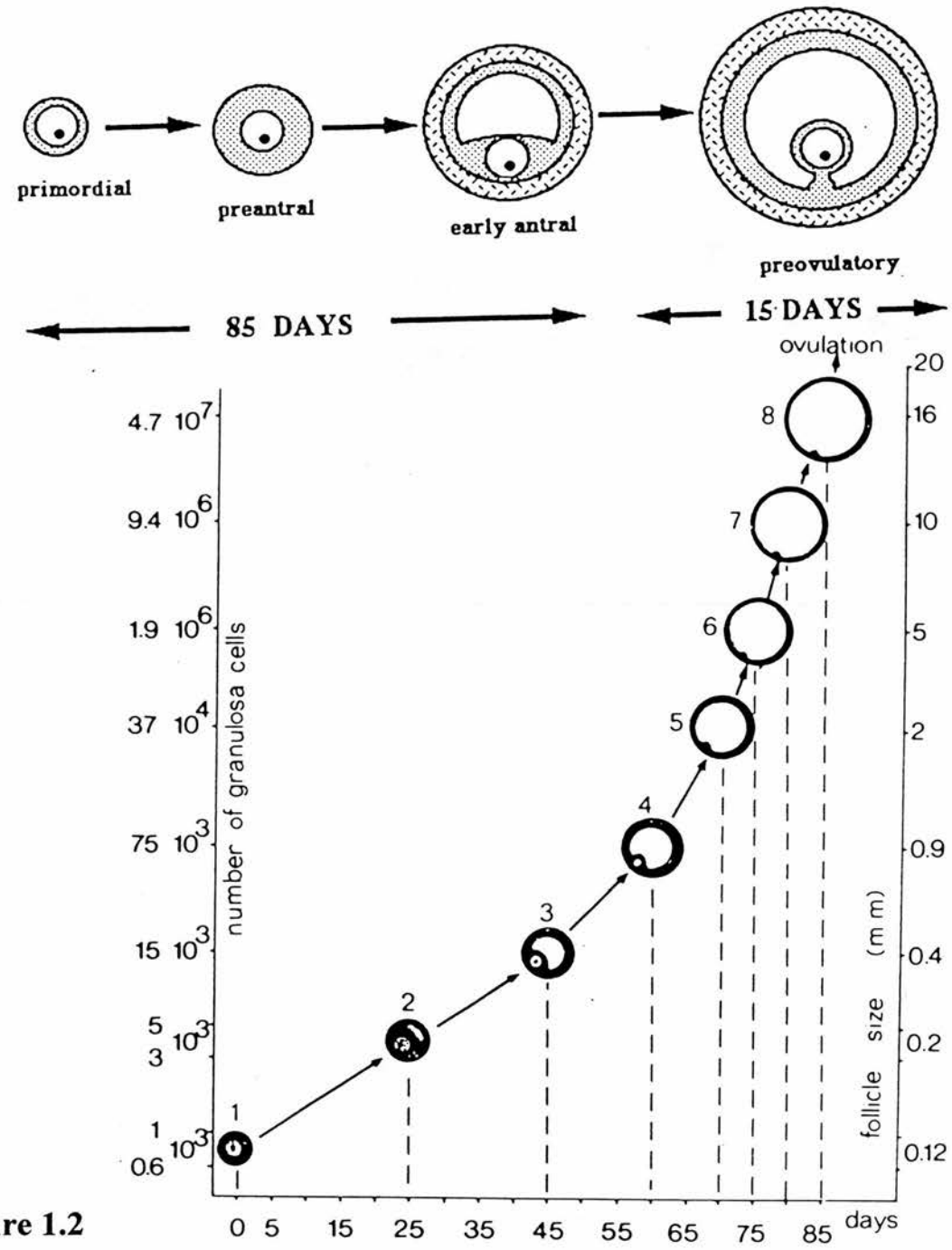

Figure 1.2

Follicular growth and development

A cartoon of the important stages of follicular growth and development. The gonadotrophin-independent preantral stage of follicular development takes 85 days. The antral stage of follicular development is gonadotrophin-dependent and is associated with rapid growth of the follicle. This stage is completed within 15 days. The growth of large follicles becomes less dependent on FSH and the granulosa cells of mature follicles develop the ability to respond to LH.

hormone (FSH). The diameter of the preovulatory follicle increases from 6.9 ±0.5 mm in the early follicular phase to 18.8 ±0.5 mm in the late follicular phase (Pache *et al.*, 1990). During this period, the mean number of granulosa cells increases from approximately 2-5 million in the early follicular phase to 50-100 million at the time of ovulation (Gougeon, 1979). This growth occurs during the follicular phase of the ovarian cycle and is completed within 15 days (Fig. 1.2).

There is a direct correlation between the size of the follicle and its blood supply (Balakier and Stronell, 1994). The preovulatory follicle becomes a highly vascular structure because of active endothelial cell proliferation in the theca cell layers (McClure *et al.*, 1994). The granulosa cells are separated from the theca cells by a basement membrane and remain avascular. The granulosa cells obtain their nutrients by diffusion, and the thickness of the granulosa cell layer in the dominant follicle is therefore limited to a maximum of seven cells (Espey and Lipner, 1994). At this stage of development, the granulosa cells of the follicle have matured to acquire the capacity to respond to the ovulatory LH surge (Richards and Midgley, 1976).

1.2.3 Ovarian Steroid Secretion

The granulosa cells and the theca cells of the antral follicle, and the luteal cells of the corpus luteum, are steroidogenic in nature. Analysis of ovarian venous blood shows that the ovary secretes oestrogens (17β-oestradiol & oestrone), progestagens (progesterone, pregnenolone & 17α-hydroxyprogesterone) and androgens (androstenedione, dehydroepiandrosterone & testosterone) (Adashi, 1994). In order to identify the cell types involved in the production of these hormones, steroid hormones have been identified and quantified in conditioned media from ovarian preparations, follicular preparations, luteal preparations and ovarian cell suspensions. These studies have identified oestrogens as the major products of the follicular granulosa cells. Androgens were found to be the major products of the thecal cells of the follicle. In contrast, luteal cells *in vitro* were able to secrete both progestagens and oestrogens (Fowler *et al.*, 1978) (Fig. 1.1).

Studies using labelled C_{21} and C_{19} precursors revealed that isolated granulosa cells were capable of producing oestrogens only when precursor hormones were present. They could not synthesise the androgens that are the immediate precursors of oestrogens in the steroidogenic pathway. In contrast, isolated theca cells produced progestagens and androgens (McNatty *et al.*, 1979). Localisation of

steroidogenic enzymes within the follicle (Conley *et al.*, 1995) have demonstrated that theca cells express 17α-hydroxylase, C17,20 lysase (17α-hydroxylase), the enzyme responsible for androgen synthesis. They do not express cytochrome P450 aromatase ($P450_{arom}$), the enzyme responsible for aromatisation of androgens to oestrogens. In contrast, granulosa cells express $P450_{arom}$ but do not have 17α-hydroxylase activity. Follicular oestrogen biosynthesis therefore requires the co-operation between granulosa cells and their thecal neighbours (Hillier, 1985).

1.2.4 Gonadotrophic Control of Ovarian Steroid Secretion

Granulosa and theca cell types are responsive to two gonadotrophins, LH and FSH (Hillier, 1994). Classic studies on hypophysectomised rats demonstrated that both LH and FSH are required for follicular oestrogen biosynthesis (Zeleznik and Fairchild Benyo, 1994). FSH receptors are located on granulosa cells and LH receptors are located on thecal cells throughout the antral stage of follicular development (Hillier, 1991). A model for gonadotrophic action within the follicle is set out in the two-cell, two-gonadotrophin hypothesis of ovarian steroid synthesis (Armstrong and Dorrington, 1979) (Fig. 1.3).

1.2.5 Maturation of Granulosa Cells

Growth of the follicle in the antral stage is dependent on gonadotrophin action. FSH binds to its cell surface receptor on the granulosa cell and activates adenylyl cyclase (Richards *et al.*, 1987). As well as stimulating cell growth, FSH induces proteins involved in steroidogenesis, such as $P450_{arom}$. This is one of the factors responsible for the marked increase in steroidogenic capacity as the follicle matures (Gougeon, 1996). As described previously, both LH and FSH are generally required for follicular steroidogenesis. However, during the late-follicular maturation of the granulosa cells, FSH induces the expression of LH receptors that are also coupled to adenylyl cyclase (Richards and Midgley, 1976). Consequently, in the preovulatory follicle, LH can regulate both androgen synthesis (in thecal cells) and aromatisation of androgen (in granulosa cells). This increases the responsiveness of the follicle in the face of FSH levels which are declining (Hillier, 1991). The decline in FSH is secondary to negative feedback actions of ovarian oestrogens and inhibins (Hillier, 1991; Groome *et al.*, 1996). Increasing oestradiol from the follicle is thought to stimulate its own formation via

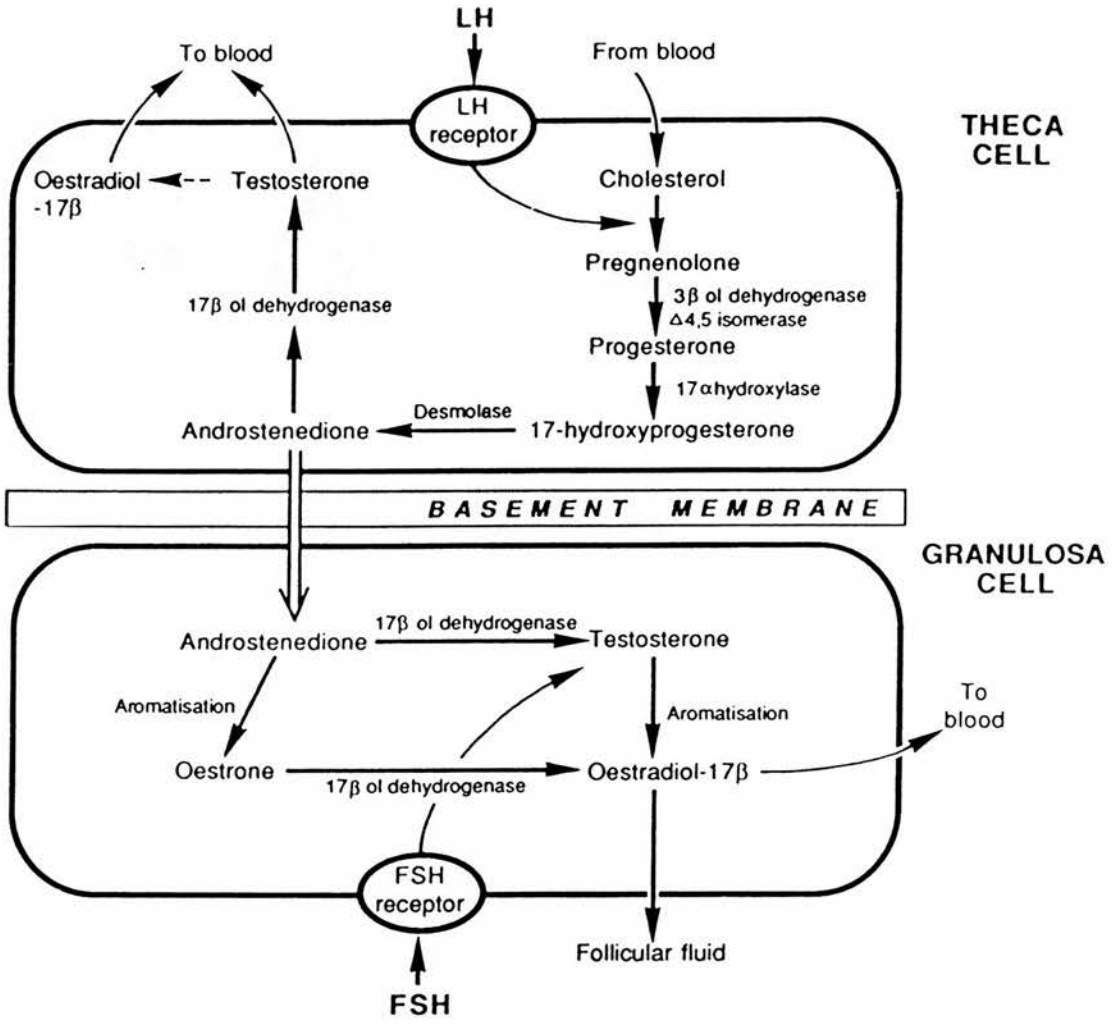

Figure 1.3

The two-cell, two-gonadotrophin hypothesis

A cartoon of the currently accepted model for follicular oestrogen synthesis. LH acts on the theca cells to stimulate androgen synthesis. These androgens diffuse to the neighbouring granulosa cells. The granulosa cells are stimulated by FSH to aromatise these androgens to oestrogens. Therefore it is thought that normal follicular oestrogen synthesis requires two cell types and two gonadotrophins.

positive feedback control of pituitary LH release (Yen, 1986). The follicle can now respond to the oestrogen-induced ovulatory LH surge (Fig 1.1).

Developmental events occurring during the follicular phase, under the control of FSH, are essential for the appropriate functioning of the corpus luteum. The preovulatory granulosa cells become the principle steroidogenic cells of the corpus luteum. Granulosa cells isolated from immature follicles are steroidogenically quiescent and require the trophic support of FSH to become steroidogenically active (Gougeon, 1996). In contrast, those collected from preovulatory follicles secreted both oestrogen and progesterone in culture conditions (Hillier *et al.*, 1981). This FSH-dependent maturation of granulosa cells during follicular development is associated with increases of messenger ribonucleic acids (RNA) (mRNAs) for the LH receptor and steroidogenic enzymes (Richards *et al.*, 1995). These are all required for the secretion of steroid hormones by the corpus luteum. Indeed, a disturbance in the normal pattern of FSH secretion during the primate follicular phase results in an abnormal luteal phase characterised by reduced progesterone production from the corpus luteum (Stouffer and Hodgen, 1980). It is likely that normal function of the corpus luteum requires a normal follicular phase.

1.2.6 Paracrine Control of Follicular Growth and Function

Although FSH and LH are the primary regulators of ovarian cellular function, it has become increasingly clear that the ovarian cellular responses to gonadotrophins can be modified by factors that are produced, and have actions, within the follicle (Hillier, 1991; Adashi, 1995; Gougeon, 1996). Many of the disparate actions of gonadotrophins on the follicle are transduced by multiple regulatory factors in a local paracrine fashion (Hillier, 1991). The local regulators which have generated the most interest are steroids, the insulin-like growth factors (IGFs) (Jones and Clemmons, 1995) and the inhibin/activin family of proteins (Aloi *et al.*, 1995).

Oestrogens are present in large concentrations within the follicular fluid. As it has been shown that oestrogens can augment FSH action in rat granulosa cells (Richards *et al.*, 1987), a role for local oestrogen in follicular development has been postulated (Hillier *et al.*, 1980). However, this effect cannot be seen in human granulosa cells *in vitro* or *in vivo* (Hillier, 1991). Androgens have been reported to augment granulosa cell aromatase activity (Hillier and deZwart, 1981)

and may promote follicular atresia (Tsafriri and Braw, 1984). The physiological importance of these actions remains unclear (Adashi, 1994). However, oestrogen, progesterone and androgen receptors have been detected on the steroidogenic cells of the primate follicle (Hild-Petito *et al.*, 1988; Suzuki *et al.*, 1994). It is therefore possible that steroids have significant intracrine or paracrine actions during follicular growth and development in the primate. At present, however, there is little information available about their role (Adashi, 1994).

There is good evidence that IGFs are involved in the augmentation of FSH effects on granulosa cell growth and differentiation (Hillier, 1991). IGFs are single chain peptides which share considerable structural and functional homologies with proinsulin (Jones and Clemmons, 1995). IGF-I and IGF-II interact with the insulin receptor, but each has a specific high-affinity receptor linked to a tyrosine kinase intracellular signalling cascade (Jones and Clemmons, 1995). The biological availability of IGFs appears to be modulated by an increasing family of specific binding proteins, the insulin-like growth factor binding proteins (IGFBPs) (Jones and Clemmons, 1995). IGFs, IGF receptors and IGFBPs can all be detected within the ovarian follicle.

Granulosa cells express IGF-I receptors and these increase after FSH treatment *in vitro* (Adashi *et al.*, 1989). IGF-I synergises with FSH to induce aromatase activity in granulosa cells, and with LH in the production of androgens (Hillier, 1991). There is evidence that ovarian cells can synthesise IGFs, and both IGF-I and IGF-II can be measured in follicular fluid (Zeleznik and Fairchild Benyo, 1994). Granulosa cells also synthesise IGFBPs and at least five different ones have been detected in follicular fluid (San Roman and Magoffin, 1992). The main IGFBP in follicular fluid was IGFBP-3. *In vitro*, FSH and LH reduced the expression of IGFBP-3 in granulosa and thecal cells respectively (San Roman and Magoffin, 1992). This complex regulatory system has not been fully dissected, but most investigators agree that the IGF system is likely to have important paracrine actions during follicular growth and development.

The inhibins and activins are proteins composed of two of three peptide subunits: an α subunit, a β_A subunit and a β_B subunit. An α subunit with a β subunit results in the formation of inhibin A or inhibin B. Two β subunits together form activin A, activin B or activin AB. Inhibins and activins were first identified on the basis of their action on pituitary FSH secretion: inhibins inhibit whereas activins stimulate (Burger and Igarashi, 1988). Activins act through specific receptors

which have now been characterised at a molecular level (Shinozaki *et al.*, 1992; Cameron *et al.*, 1994). An intensive search for specific inhibin receptors has, to date, proved unsuccessful. A specific binding protein, known as follistatin, has been described which will bind inhibin and affect its biological bioavailability (Ying, 1988). The proportions of activins and inhibins synthesised, are likely to have major effects on their net biological action (Findlay, 1993).

Changing expression of inhibin/activin subunits during follicular growth suggests that they may have a paracrine role during folliculogenesis (Fraser *et al.*, 1993; Roberts *et al.*, 1993; Fraser and Lunn, 1993; Findlay, 1993; Fraser *et al.*, 1995a). Inhibins and activins share structural homology with a family of growth factors, which includes transforming growth factor (TGF) β, that are involved in embryogenesis, tissue repair and remodelling (Roberts *et al.*, 1988). Early antral follicles primarily express the β_B subunit and preovulatory follicles express the β_A and α subunits. This is likely to explain the changing expression of inhibin A and inhibin B during the menstrual cycle (Groome *et al.*, 1996). This also suggests that activins may be involved in the early stages of antral follicular development and that inhibins may be involved in the final stages of preovulatory development (Zeleznik and Fairchild Benyo, 1994).

Secretion of inhibin/activin is regulated by gonadotrophins and sex steroids *in vitro* (Hillier, 1991). FSH induces inhibin production from granulosa cells and this is augmented by the presence of androgen (Hillier, 1991). Inhibin itself induces potent and selective stimulation of human thecal androgen synthesis *in vitro* (Hillier *et al.*, 1991a). In contrast, activin antagonises the stimulatory effect of LH on thecal androgen production (Hillier *et al.*, 1991a). Inhibin and activin may also have effects on cellular proliferation within the follicle. Inhibin has some inhibitory effects on ovarian cellular proliferation (Matzuk *et al.*, 1992), whereas activin can stimulate cellular proliferation *in vitro* (Rabinovici *et al.*, 1990; Li *et al.*, 1995). It is likely that the differential effects of activins and inhibins on the follicle (Miro and Hillier, 1992), and their different temporal expression, are important during folliculogenesis. At present, however, more work is required into the roles of these proteins in ovarian function.

Other molecules have been implicated as having potential roles as paracrine regulators of follicular development. These include growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), TGFα and TGFβ. EGF, bFGF and TGFα have been shown to promote proliferation *in vitro*,

and all these molecules can both positively and negatively affect steroid synthesis (Gougeon, 1996). In addition, various cytokines, that are primarily white cell products, have been shown to affect steroidogenesis (Adashi, 1990). Tumour necrosis factor (TNF) α , interleukins (IL) 1,2 and 6 and interferons (IFN) α, β and γ have all been shown to inhibit gonadotrophin-induced steroidogenesis *in vitro*. (Gougeon, 1996). However, these molecules tend to increase prior to ovulation and their primary role may be as regulators of ovulation (Espey, 1994), rather than steroidogenesis. Although all the above molecules can be detected in the ovary, the physiological implications of their actions is not yet clear.

To recap, the dominant follicle has two distinct steroidogenic cell layers. Steroidogenesis is dependant on pituitary gonadotrophins and relies on an interaction between the steroidogenic cell layers. Cellular proliferation and steroidogenesis can be modified by a range of local paracrine regulators. Although the exact role of these regulators is not clear, they include steroids, growth factors, cytokines and other peptides. As follicles grow and mature, oestrogen secretion increases and a dominant follicle is selected. When the dominant follicle is fully mature, the secreted oestrogen has prepared the endometrium for pregnancy. The oocyte is now ready for release and, therefore, the next process to be considered is ovulation.

1.3 Ovulation

The next stage of the ovarian cycle to be considered is ovulation. Ovulation is induced by LH from the pituitary gland in response to increasing ovarian steroid synthesis from the dominant follicle. This gonadotrophin surge induces a series of changes in various follicular compartments cumulating in the release of a fertilisable ovum and the transformation of the follicle into the corpus luteum (Espey and Lipner, 1994). The ovulatory response can be divided into three main components: reactivation of oocyte maturation, follicular rupture, and altered steroidogenic capacity of the granulosa cells (luteinisation) (Tsafriri and Dekel, 1994).

1.3.1 Oocyte Reactivation

Ovulation is induced by LH acting on specific LH receptors. These LH receptors are localised to the steroidogenic cells of the dominant follicle. Therefore steroidogenic cell factors must be involved in the initiation of each element of the ovulatory response. As inhibitors of RNA and protein synthesis have been shown to block ovulation (Tsafriri and Dekel, 1994), it is clear that the ovulatory process involves the stimulation of new transcription and translation. The oocyte does not express LH receptors, so its reactivation in response to the LH surge must depend on signals released from the LH responsive cells.

Oocytes mature spontaneously when removed from granulosa cell contact (Espey and Lipner, 1994). This maturation can be prevented by addition of cyclic adenosine monophosphate (cAMP) or adenylyl cyclase to the oocyte. As the oocyte contains little natural adenylyl cyclase activity (Tsafriri and Dekel, 1994), the source of the cAMP required to delay maturation is likely to be the granulosa cells, through their direct cytoplasmic connections (Weiss *et al.*, 1976). The high granulosa cell cAMP, stimulated during the LH surge, breaks down intracellular connections between granulosa cells, and between granulosa cells and the oocyte (Albertini and Anderson, 1974). The oocytes exposure to cAMP therefore falls. This results in altered protein phosphorylation within the oocyte and it reenters the cell cycle (Tsafriri and Dekel, 1994).

1.3.2 Follicular Rupture

The LH surge, by acting on the steroidogenic cells of the follicle, also results in follicular rupture. Morphological studies demonstrate a reaction in the follicle wall which is akin to an acute inflammatory response (Espey, 1994). The exact mechanisms of follicular rupture are not fully understood, but cellular changes occur in cell layers which do not express LH receptors. This suggests that the steroidogenic cells must respond to the LH surge by influencing factors which act on the outer layers of the follicle (Espey and Lipner, 1994). It is not clear what these factors are, but eicosanoids and steroids have been implicated (LeMaire and Marsh, 1975; Priddy and Killick, 1993). The net result of these factors is to stimulate and augment the early stages of an inflammatory reaction (Espey, 1980) in the follicle wall.

The inflammatory reaction stimulates cascades leading to serine protease activity. Collagenases are synthesised and then activated by these proteases (Reich *et al.*, 1985; Reich, 1991), which soften the follicular wall at the stigma. As the wall becomes softer, fibroblasts become more motile and active, and migrate away from the stigma (Espey and Lipner, 1994). The follicle wall becomes thinner and more avascular, the internal pressure of the follicular fluid causes ballooning of this portion of the wall, the epithelial cells slough off, and the follicle ruptures (Espey and Lipner, 1994). The cumulus-oocyte complex is released over many minutes in a wave of follicular fluid. Ovulation is also associated with a change in the synthetic capacity of the granulosa cells (Tsafriri and Dekel, 1994). The collapsing follicle, with its luteinised granulosa cells, becomes the corpus luteum.

1.3.3 Luteinisation

Luteinisation is the process where the granulosa cells of the preovulatory follicle acquire the machinery to produce progesterone, in preparation for their role in the corpus luteum. Until the LH surge, the steroidogenic role of granulosa cells is largely limited to the aromatisation of the androgen products from the thecal cells (Hillier, 1994). FSH-induced maturation of the granulosa cells, with the LH surge, induces the expression of the steroidogenic enzymes, P450 cholesterol side-chain cleavage ($P450_{scc}$) and 3β-hydroxysteroid dehydrogenase (3β-HSD), that catalyse the synthesis of progesterone from cholesterol (*vide infra*) (Miller, 1988). The granulosa cells change their appearance during this process. They become larger (increasing in size by 250%), develop intracellular granules, and become rich in smooth and rough endoplasmic reticulum, and mitochondria with tubular cristae (Zeleznik and Fairchild Benyo, 1994).

This process can be mimicked by the introduction of cAMP into the granulosa cells, and is not affected by inhibitors of prostaglandins and other mediators of acute inflammation (Tsafriri and Dekel, 1994). Granulosa cells will spontaneously luteinise and produce progesterone in culture (Hillier *et al.*, 1981). This means that the follicular environment has an inhibitory effect on luteinisation, which is removed by the LH surge. Luteinisation induced by removing the granulosa cells from the follicular environment, does not involve large increases in intracellular cAMP, nor can it be prevented by incubating the cells in granulosa cell conditioned media, or follicular fluid (Espey and Lipner, 1994). Although the molecular events causing luteinisation are not clear, it may be that the breakdown

of granulosa cell communications with each other, induced hormonally by the LH surge and mechanically during removal from the follicle, is involved.

The follicle has now grown, matured and ruptured, releasing a fertilisable oocyte. The collapsing follicle still contains an avascular layer of granulosa cells surrounded by highly vascular thecal cells. These granulosa cells, however, have luteinised and are able to synthesise and secrete progesterone, as well synthesising oestrogens by aromatisation of thecal androgens. They have lost their FSH receptors and now are controlled by the major gonadotrophin involved in the luteal phase of the ovarian cycle: LH. These follicular remnants are transformed into the corpus luteum.

1.4 The Corpus Luteum

We have now reached the luteal phase of the ovarian cycle. This phase is dominated by the corpus luteum. This dynamic gland is formed from the follicular remnants, secretes large amounts of progesterone, and then regresses in the absence of pregnancy. The function of the corpus luteum and the mechanisms responsible for its regression and 'rescue' during pregnancy will be discussed in detail. However, the first aspects of the corpus luteum to be covered are its formation, structure and cellular composition.

1.4.1 Formation of the Corpus Luteum

The transformation of the dominant follicle into the corpus luteum is still not understood at a molecular level. The early work of Corner (1956) was instrumental in defining the structural and morphological characteristics of the human corpus luteum throughout the luteal phase. These elegant descriptions give snap-shots of the human corpus luteum at different time points in its life-cycle. The dynamic nature of the formation of the corpus luteum, however, required comprehensive studies of carefully controlled rat models. Although the control of the rat corpus luteum differs from that of the primate (Niswender and Nett, 1994), there is no reason to suggest that the cellular events surrounding luteinisation and the formation of the rat corpus luteum differ from those in the human (Behrman *et al.*, 1993).

Following the LH surge, the basement membrane between the theca interna and the granulosa cells begins to break down. The theca engorges with blood during this process, becomes oedematous, and begins to form invaginations into the avascular granulosa cells (Pederson, 1951). There is extravasation of red blood cells into the antral cavity, and this fills with blood. Blood vessels from the theca invade the follicular antral space and, under the influence of angiogenic factors, new, and more extensive, vascular structures develop (Reynolds *et al.*, 1992; McClure *et al.*, 1994; Redmer and Reynolds, 1996). Both the theca and granulosa cell layers undergo hypertrophy at this time. Theca cells invaginate into the granulosa cell layer, but unlike other species (Niswender *et al.*, 1994), remain separate from the granulosa-lutein cells. Fibroblasts also invade into the granulosa-lutein cell layer to give structural support.

The human corpus luteum has a central core of fibrin clot (Fig. 1.4). Surrounding this is the thickened granulosa-lutein cell layer with numerous infoldings. This cell layer is highly vascular and each granulosa-lutein cell is thought to abut a vascular endothelial cell (Dharmarajan *et al.*, 1985). The theca derived cells are easily distinguishable from the granulosa derived cells in the human corpus luteum (Sasano *et al.*, 1989; Rodger *et al.*, 1995). They form small clumps at the periphery of the granulosa-lutein cells (Fig. 1.4). The theca-lutein cells invaginate the granulosa-lutein cell layer along vascular fibrous infoldings which gives the gland a spoke and wheel appearance (Corner, 1956). The steroidogenic cells are surrounded by a dense fibrous stroma containing blood vessels, fibroblasts, immune cells and extracellular matrix (ECM) (Behrman *et al.*, 1993) (Fig. 1.4).

1.4.2 Composition of the Corpus Luteum

It is clear that the corpus luteum contains several different types of cell. These are all likely to be involved in luteal function. Firstly, the corpus luteum contains cells whose primary function is that of steroidogenesis. These cells will contain the steroidogenic enzymes necessary to synthesise not only progesterone but other steroid hormones such as oestrogen (Miller, 1988), and peptide hormones such as inhibin A (Illingworth *et al.*, 1991; Groome *et al.*, 1996) and relaxin (Sherwood, 1994). The corpus luteum is the most vascular tissue in the body, with a blood supply per unit mass, eight times that of the kidney (Bruce and Moor, 1976; Ford *et al.*, 1982). Endothelial cells, lining blood vessels and capillaries, are therefore likely to form a large component of the gland. It has been estimated that over fifty

Figure 1.4

Histological structure of the corpus luteum

Haematoxylin and eosin stained section of a human corpus luteum from the mid-luteal stage of the luteal phase. The central portion of the corpus luteum contains extravasated red blood cells and fibrin clot (C). Surrounding this are the granulosa-lutein cells (G). On the periphery of the gland, in separate clumps, are the smaller theca-lutein cells (T). The fibrous connective tissue (F) invaginates the gland in a spoke and wheel appearance. The corpus luteum is contained in a stromal capsule (S) containing blood vessels, fibroblasts and extracellular matrix (BV). Scale Bar =200 μm.

percent of cells in the corpus luteum are endothelial cells (Reynolds *et al.*, 1992; Zheng *et al.*, 1994) and that each steroidogenic cell is surrounded by a network of capillaries (Dharmarajan *et al.*, 1985).

Another group of cells in the corpus luteum are those whose primary function is likely to be structural. These will include fibroblasts and pericytes in the gland capsule and around blood vessels (Lei *et al.*, 1991). Lastly, there are the migratory cells such as macrophages, monocytes and leukocytes. Their presence has been clearly described in corpora lutea (Brännström *et al.*, 1994a) and they have been implicated in the regulation of luteal integrity (Brännström and Norman, 1993). All these cells are contained within the ECM. This serves as the glue holding the corpus luteum together. However as well as having a major structural role, the ECM probably contributes essential elements to the functional activity of the gland (Salamonsen, 1996; Hulboy *et al.*, 1997).

1.4.3 Steroidogenic Cells

There are two types of steroidogenic cells in the corpus luteum. Those classified as small (theca-lutein), with a thecal origin, and those classified as large (granulosa-lutein), with a granulosal origin. They can be distinguished on the basis of their size, morphology and functional characteristics. Granulosa-lutein cells continue to express $P450_{arom}$ and theca-lutein cells continue to express 17α-hydroxylase (Sasano *et al.*, 1989). Although these two cell types can be found in most mammalian species, they are usually found intermixed together (Niswender and Nett, 1994). In the human however, they remain in discrete clumps separated from each other. In the human, there are about twice as many large as small luteal cells (Lei *et al.*, 1991). This pattern does not appear to change throughout the luteal phase (Lei *et al.*, 1991).

Although theca cell hyperplasia occurs at the time of the LH surge, luteinisation is associated with terminal cellular differentiation. Studies in rats have convincingly demonstrated that steroidogenic cells in corpora lutea do not incorporate [^{3}H] thymidine into nuclei, a measure of DNA synthesis, whereas such incorporation is readily apparent in follicular granulosa cells (Hirschfield, 1984). In addition, the mitotic index of granulosa cells decreases markedly after the LH surge and mitotic figures in human corpora lutea are rare (Corner, 1956; Gougeon, 1996). Recent studies using immunohistochemical assessment of nuclear proliferation markers have elegantly shown that cellular proliferation in the primate corpus luteum is

largely limited to endothelial cells (Christenson and Stouffer 1996a; Rodger *et al.*, 1997). In addition luteal 'rescue' is not associated with an increase in cellular proliferation. (Rodger *et al.*, 1997). The steroidogenic cells of the corpus luteum have one fate: death when their function is served.

The granulosa-lutein and theca-lutein cells express LH receptors, but not FSH receptors. They both express the steroidogenic enzymes responsible for the synthesis of progesterone (Miller, 1988). There is little evidence, in the human, to show functional differences in the steroidogenic nature of the theca-lutein and granulosa-lutein cells. In the ruminant, however, it is thought that progesterone production from the small cells is LH responsive, and that from the large cells in largely autonomous (Niswender *et al.*, 1985). As the primate corpus luteum is absolutely dependant on the trophic support of LH (Hutchison and Zeleznik, 1984), this is unlikely to be the case in the primate. As luteal oestrogen production parallels that of progesterone (Lenton and Woodward, 1988), and requires the steroidogenic capacity of both cell types (Sasano *et al.*, 1989; Sanders and Stouffer, 1997), it is likely that both cell types are equally active within the primate corpus luteum. However, detailed studies of these cells in the primate are not yet available.

1.4.4 Vascular Endothelial Cells

The granulosa cell layer of the dominant follicle contains no blood vessels. These cells are nourished by diffusion of nutrients from the blood vessels in the theca cell layer (Gougeon, 1996). The corpus luteum has one of the highest blood flow rates in the body (Bruce and Moor, 1976; Ford *et al.*, 1992). In the rat, over 60% of the luteal cell surface abuts a capillary and the remainder faces the interstitial space which extends to the surface of capillaries (Dharmarajan *et al.*, 1985). In both ruminants (Lei *et al.*, 1991) and primates (Reynolds *et al.*, 1992), over half of the cells in the mature corpus luteum are endothelial cells. The steroidogenic cells of the corpus luteum of granulosa cell origin are found within a luxurious network of blood vessels. Luteinisation is therefore associated with intense angiogenic activity.

Blood vessels invade the granulosa from the theca during dissolution of the membrana granulosa. New vessel growth involves several distinct phases (Folkman, 1985; Reynolds *et al.*, 1992). The first step is the dissolution of the endothelial cell basement membrane. This is likely to involve the action of

proteolytic enzymes. Although both matrix metalloproteinases (MMPs) (Tsang *et al.*, 1995) and serine proteases (Liu *et al.*, 1996) have been detected in the corpus luteum, their role in luteal angiogenesis has not yet been established. The next stage of angiogenesis is the proliferation and migration of endothelial cells (Reynolds *et al.*, 1992). It is clear that in ruminants (Zheng *et al.*, 1994), non-human primates (Christenson and Stouffer, 1996a), and women (Rodger *et al.*, 1997), there is marked endothelial cell proliferation in the early-luteal phase. Several factors have been identified as inducing trophic and proliferative responses on endothelial cells, to stimulate angiogenesis.

There is no single angiogenic factor which is thought to be wholly responsible for angiogenesis in the developing corpus luteum. Many growth factors and cytokines, that can be detected in the corpus luteum, have been shown to have angiogenic activity (Klagsbrun and D'Amore, 1991; Gordon *et al.*, 1996). EGF (Huang *et al.*, 1995; Tamura *et al.*, 1995), platelet derived growth factor (PDGF) (Bagavandoss and Wilks, 1991), IGF-1 (Jones and Clemmons, 1995), TGFα and TGFβ (Behrman *et al.*, 1993; Tamura *et al.*, 1995), TNFα, IL-1 and IL-6 (Bagavandoss and Wilks, 1991) are all thought to influence luteal angiogenesis. However, experimental and observational studies *in vitro* suggest that bFGF and vascular endothelial growth factor (VEGF) are the principle luteal endothelial cell mitogens (Reynolds *et al.*, 1992; Redmer and Reynolds, 1996; Redmer *et al.*, 1996). Indeed, although there appears to be redundancy in many of these angiogenic factors, it has recently been shown that VEGF is essential for corpus luteum angiogenesis (Ferrara *et al.*, 1998).

1.4.5 Extracellular Matrix

The ECM constitutes a large part of the shape and volume of the corpus luteum. It serves as the glue which holds the cellular components of the corpus luteum together and forms the pathways for migration of transient cells. It consists of collagen, proteoglycans, laminin, fibronectin and other components. As well as a structural role, it is now clear that the ECM also has dynamic functions. It influences cell proliferation, cell death and cell differentiation and forms a repository for biologically active growth factors (Hulboy *et al.*, 1997). The ECM clearly has to be in a dynamic state during the tissue remodelling (Woessner, 1991) that occurs during the growth, development and atrophy of all tissues, including the corpus luteum (Hulboy *et al.*, 1997).

It is therefore clear that agents that modify the ECM, have the potential to affect a wide range of physiological and pathological processes (Matrisian, 1990). Remodelling of connective tissue requires both breakdown and resynthesis of ECM components. The degradation of ECM proteins can be effected by a variety of proteolytic enzymes. The MMPs are the key enzymes involved in ECM remodelling (Matrisian, 1990; Woessner 1991; Birkedal-Hansen, 1995; Hulboy *et al.*, 1997). They are the only secreted enzymes capable of denaturing fibrillar collagens, they are tightly controlled, but active under physiological conditions, and are localised to areas of active tissue remodelling (Hulboy *et al.*, 1997). MMPs can be detected in the corpus luteum (Endo *et al.*, 1993a; Tsang *et al.*, 1995).

1.4.6 Immune Cells

Other notable cells of the corpus luteum are the immune cells. Macrophages are prominent cells in the corpora lutea of several species (Wang LJ *et al.*, 1992; Brännström *et al.*, 1994b; Hehnke *et al.*, 1994; Naftalin *et al.*, 1997). Macrophages appear to increase in number throughout the luteal phase (Best *et al.*, 1996) and are particularly present in the regressing corpus luteum at the time of menstruation (Wang LJ *et al.*, 1992; Brännström *et al.*, 1994a). Much less is known about other immune cells in the human corpus luteum. It is likely that there are T-lymphocytes, neutrophil polymorphonuclear leukocytes, eosinophils and monocytes (Brännström *et al.*, 1994a; Best *et al.*, 1996) in corpora lutea. It is likely that these immune cells serve important roles in the regulation of luteal function. They are fundamental sources of cytokines, that are increasingly recognised as potential local regulators of luteal function (Brännström and Norman, 1993).

1.5 Steroidogenesis

The main function of the corpus luteum is steroidogenesis, and the principle secreted steroid hormone is progesterone. Indeed, the progesterone the corpus luteum secretes is fundamental to its function. In the absence of any ovarian activity, oestrogen followed by progesterone supplementation adequately prepares the endometrium for implantation (Hodgen, 1983). In anovulatory cycles, exogenous progesterone replacement and withdrawal will mimic the normal

uterine environment and menstrual response (Li *et al.*, 1991a). In the absence of a corpus luteum, in donor oocyte pregnancies, progesterone supplementation alone will maintain an early pregnancy (Lutjen *et al.*, 1984). Luteectomy in the first few weeks of pregnancy, will cause miscarriage (Csapo *et al.*, 1973). Supplementation with exogenous progesterone after luteectomy will maintain the pregnancy (Csapo *et al.*, 1973). It is clear that progesterone secretion is the primary function of the corpus luteum.

1.5.1 Progesterone Synthesis

The human corpus luteum secretes huge amounts of progesterone. At its peak of activity, it produces up to 25 mg of progesterone each day (Strauss *et al.*, 1981). The building block for progesterone and other steroid hormones is cholesterol. Although the corpus luteum can synthesise cholesterol *de novo*, the available evidence suggests luteal cholesterol is derived directly from the plasma, and the mobilisation of intracellular stores (Strauss *et al.*, 1981; Behrman *et al.*, 1993). Cholesterol is transported in the plasma as lipoprotein complexes (Goldstein and Brown, 1977). Although cholesterol is present in both low-density lipoproteins (LDL) and high-density lipoproteins (HDL), in the human ovary it seems that the main source of cholesterol is LDL (Soto *et al.*, 1984).

LDL receptors on the membranes of luteal cells bind LDL. The bound receptors then aggregate into coated pits on the cell surface and are internalised (Strauss *et al.*, 1981). The internalised complexes associate with lysozymes, and cholesterol is liberated (Stocco and Clark, 1996). This cholesterol is either used immediately, for steroidogenesis, or esterified and stored in lipid droplets within the cell (Strauss *et al.*, 1981). Steroidogenic cells in the corpus luteum can clearly be seen to contain lipid droplets at the ultrastructural level (Gillim *et al.*, 1969). It is the stored lipid which is partly thought to be responsible for the characteristic colour and texture of the corpus luteum. Expression of LDL receptors has been shown to be stimulated by gonadotrophins (Talavera and Menon, 1989). This means that one of the luteotrophic effects of gonadotrophin is stimulation of the availability of cholesterol substrate. However, it is generally accepted that the rate limiting stages of progesterone production involve the enzymatic processing, rather than the supply, of cholesterol (Stocco and Clark, 1996).

The sex steroid synthetic pathway is well defined (Figure 1.5). Cholesterol is modified by specific steroidogenic enzymes acting in series. The first step is the

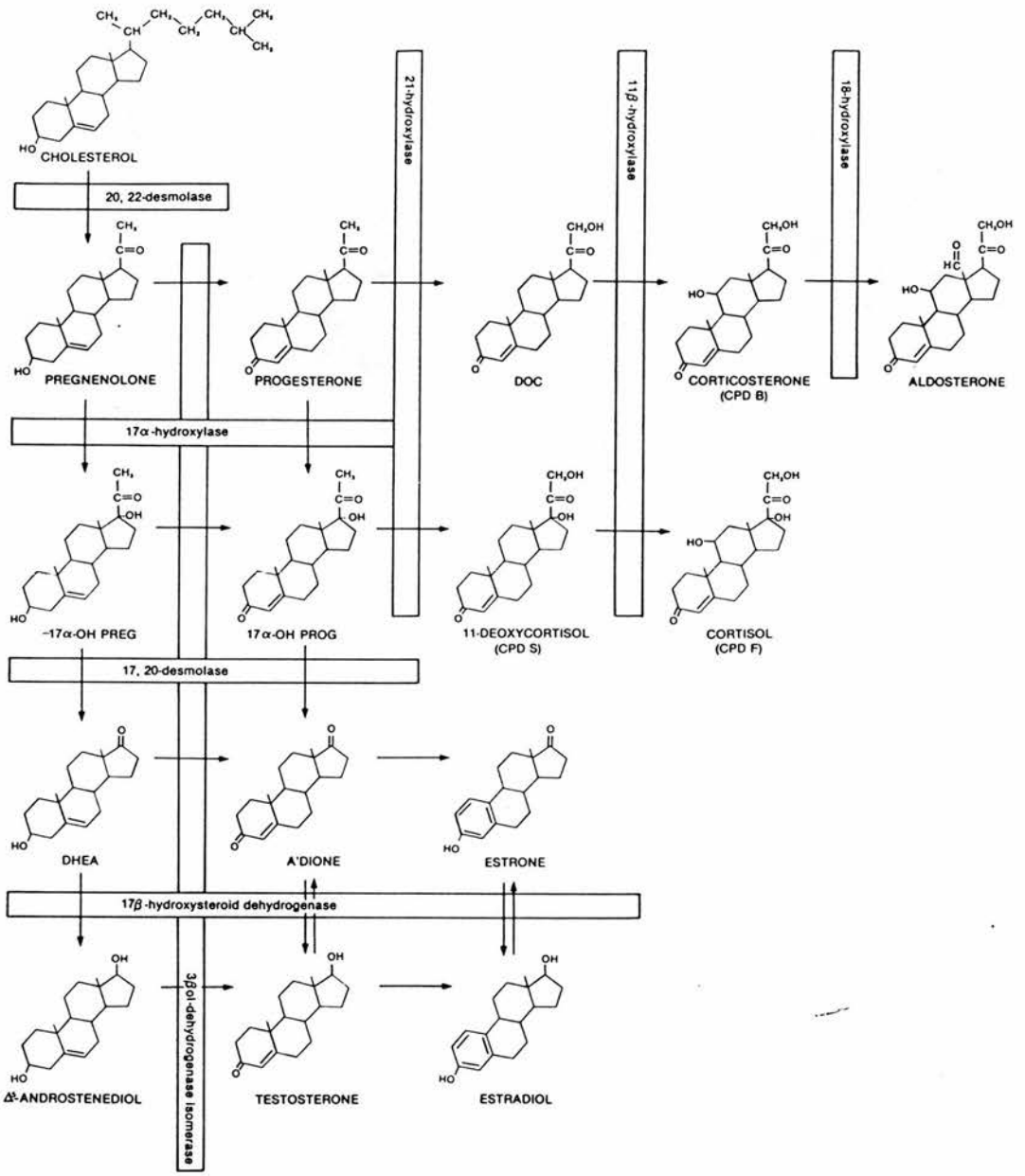

Figure 1.5

The synthetic pathway for sex steroid hormones

A cartoon of the synthetic pathway, and the enzymes involved, in the production of sex steroids. Most reactions occur in the cell cytosol and endoplasmic reticulum, but the conversion of cholesterol to pregnenolone is catalysed by an enzyme complex located on the inner mitochondrial membrane. This involves the action of a specific cholesterol transport protein.

conversion of cholesterol to pregnenolone. This is carried out by a $P450_{scc}$ (Simpson and Boyd, 1967). However, the $P450_{scc}$ enzyme complex is located on the inner mitochondrial membrane (Stocco and Clark, 1996), and the cholesterol is located within the cell cytosol. In order to gain access to the inner mitochondrial space, the hydrophobic cholesterol has to cross the fluid space between the mitochondrial membranes. It is clear that this hydrophilic space is a barrier to the hydrophobic cholesterol and that carrier molecules are necessary to transport the cholesterol to the $P450_{scc}$ complex. *De novo* protein synthesis is necessary for this step (Garren *et al.*, 1965), implying that specific carrier proteins are involved in cholesterol transport.

Several different carriers have been implicated in this process, including sterol carrier protein-2 (SCP-2) and other molecules (Hall, 1985; Stocco and Clark, 1996). However, it now seems likely that the best candidate molecule for the cholesterol transport protein, is the recently characterised steroidogenic acute regulatory protein (StAR) (Clark *et al.*, 1994; Stocco and Clark, 1996). StAR has been shown to be absolutely required for regulated steroidogenesis (King *et al.*, 1995; Lin *et al.*, 1995; Waterman, 1995) and it has been detected in ovine (Juengel *et al.*, 1995), bovine (Hartung *et al.*, 1995), and primate corpora lutea (Kiriakidou *et al.*, 1996). Other carrier molecules must also be physiologically relevant, as StAR is not involved in placental progesterone production (Lin *et al.*, 1995). However, it seems likely that StAR expression is of fundamental importance in luteal steroidogenesis (Stocco and Clark, 1996)

The conversion of cholesterol to pregnenolone is the rate limiting step in steroidogenesis (Hall, 1985). The rate of this step is dependent on StAR (King *et al.*, 1995). It is therefore likely that StAR expression and activity is the rate limiting step of acutely regulated steroidogenesis. In the cytosol, in association with mitochondria, pregnenolone is converted into progesterone by the action of 3β-HSD. Progesterone itself can be converted into oestradiol via an androgen intermediate. The enzyme 17α-hydroxylase converts progesterone into androstenedione, which in turn is converted to 17β-oestradiol by $P450_{arom}$ (Strauss and Miller, 1991).

1.5.2 Other Steroid Products of the Corpus Luteum

As well as secreting progesterone, the primate corpus luteum also secretes oestrogen (Lenton and Woodward, 1988; Zeleznik and Fairchild Benyo, 1994).

Oestrogen synthesis in the corpus luteum appears to involve the same ontogeny as oestrogen biosynthesis in the follicle (Sanders and Stouffer, 1997). Both granulosa-lutein cells and theca-lutein cells are involved in its synthesis. Like the follicle, the theca-lutein cells are responsible for the synthesis of androgen substrates and the granulosa-lutein cells are responsible for their aromatisation to oestrogens. In the corpus luteum, however, it is probable that the progesterone required for androgen biosynthesis is obtained from both the theca-lutein and the granulosa-lutein cells. It is also likely that the LH receptors, now present on both these cell types, are wholly responsible for the oestrogen synthesis. The role for FSH is not clear, there are no FSH receptors in the corpus luteum, and oestrogen biosynthesis occurs when only LH is replaced in the luteal phase (Hutchison and Zeleznik, 1984).

1.5.3 Non-Steroid Products of the Corpus Luteum

In addition to steroid hormones, the primate corpus luteum also synthesises and releases a variety of protein hormones including relaxin (Sherwood, 1994), oxytocin (Khan-Dawood *et al.*, 1984), inhibin and activin (Fraser and Lunn, 1993). Relaxin is a protein hormone with structural similarities to insulin and IGFs (Sherwood, 1994). Relaxin protein and mRNA can be detected in the steroidogenic cells of the corpus luteum (MacLennan *et al.*, 1991; Stoelk *et al.*, 1991; Sherwood, 1994). Indeed, the development of sensitive relaxin immunoassays has demonstrated that it is secreted into the peripheral circulation during the luteal phase of the cycle (Sherwood, 1994; Duffy *et al.*, 1995). Relaxin has been shown to have effects on ligaments, the cervix, uterus and vagina (Sherwood, 1994), and it has a clear role during gestation. However, to date, the biological endocrine or paracrine role of luteal relaxin during the menstrual cycle has not been determined.

Oxytocin and its carrier protein have been localised to the steroidogenic cells of the primate corpus luteum (Khan-Dawood and Dawood, 1983). Ovarian venous oxytocin concentrations in the luteal phase are higher than in the peripheral circulation (Dawood and Khan-Dawood, 1986), and are reduced after luteectomy (Khan-Dawood *et al.*, 1988). In addition, the oxytocin gene has also been shown to be expressed in primate corpora lutea (Ivell *et al.*, 1990; Khan-Dawood *et al.*, 1995). However, the pituitary gland is the major source of oxytocin in the circulation, and there is no evidence for any endocrine effects of luteal oxytocin.

Indeed, there is no evidence for an endocrine effect of physiological levels of oxytocin on the corpus luteum (Auletta and Flint, 1988). However, there are oxytocin receptors within the corpus luteum (Khan-Dawood *et al.*, 1993) and some authors have postulated a paracrine action of oxytocin in the primate corpus luteum (Khan-Dawood, 1997).

In the primate, inhibin production is a characteristic function of the corpus luteum (Illingworth *et al.*, 1991). Indeed, the corpus luteum produces substantially more inhibin than does the follicle, and concordant changes in circulating inhibin and progesterone are evident throughout the menstrual cycle (McLachlan *et al.*, 1987). Recently, it has been clearly shown that the human corpus luteum secretes large amounts of inhibin A and pro-α-C subunit (Groome *et al.*, 1995; Groome *et al.*, 1996). Indeed, mRNAs for inhibin subunits are highly expressed in the primate corpus luteum (Fraser *et al.*, 1993; Roberts *et al.*, 1993). One role of luteal inhibin secretion may be to augment the inhibition of FSH secretion, and inhibit antral follicular growth, during the luteal phase of the ovarian cycle (Fraser and Lunn, 1993; Baird and Smith, 1993). The decrease of inhibin secretion at luteal regression (Illingworth *et al.*, 1991), may cause the elevation of serum FSH levels that begins at menses and is responsible for subsequent follicular development.

1.5.4 Systemic Regulators of Steroidogenesis

The corpus luteum is not an autonomous gland. In the absence of systemic gonadotrophic support, its function rapidly ceases (Hutchison and Zeleznik, 1984). LH from the pituitary is the principal regulator that controls luteal function. Removal of the pituitary surgically, or the gonadotrophins pharmacologically, results in rapid loss of luteal function (Asch *et al.*, 1982; Fraser *et al.*, 1986). Replacement of LH or hCG alone maintains normal luteal function (Thau *et al.*, 1983; Hutchison and Zeleznik, 1984). In addition, LH can directly increase progesterone output from isolated luteal cells and luteal tissue (Stouffer *et al.*, 1977; Dennefors *et al.*, 1982). The steroidogenic cells of the corpus luteum express specific receptors to LH (Ravindranath *et al.*, 1992a).

LH binds to specific high affinity receptors on luteal cells (Cole *et al.*, 1973; Bramley *et al.*, 1987). The LH receptor has been cloned and it belongs to the family of transmembrane GTP binding protein (G-protein)-coupled receptors (Minegishi *et al.*, 1990). LH receptors have seven transmembrane domains, a large glycosylated extracellular N-terminal domain and a smaller C-terminal

intracellular domain (Segaloff and Ascoli, 1993) (Fig. 1.6). Binding of LH activates G-proteins and stimulates the enzyme adenylyl cyclase to produce cAMP (Niswender and Nett, 1994). Other molecules and enzymes, including calcium ions, phospholipase C, diacylglycerol (DAG), inositol triphosphate (ITP) and protein kinase C (PKC), have also been implicated in the second messenger pathways activated by the LH receptor (Cooke *et al.*, 1989; López Bernal *et al.*, 1995). However, it appears that cAMP is the primary second messenger molecule and that responsible for the stimulation of steroidogenesis (Marsh, 1976; Dennefors *et al.*, 1982; Rojas *et al.*, 1989)

Although LH is absolutely required to maintain luteal function, circulating LH levels are low throughout the luteal phase and do not parallel the output of progesterone (Ellinwood *et al.*, 1984). Although there are changes in the LH receptor (Ravindranath *et al.*, 1992a; Nishimori *et al.*, 1995) and adenylyl cyclase (Eyster *et al.*, 1985; Rojas *et al.*, 1989) throughout the luteal phase, these do not always parallel progesterone secretion. It is therefore likely that the action of LH on steroidogenic luteal cells is modulated in a paracrine fashion by local regulators of steroidogenesis.

1.5.5 Local Regulators of Steroidogenesis

Just as local paracrine regulators of steroidogenesis have been described in the ovarian follicle (Gougeon, 1996), they have been described in the corpus luteum. IGFs, inhibins and activins, steroids, eicosanoids, cytokines, growth factors and other molecules (Tsafiri and Adashi, 1994) have been reported as potential local regulators of luteal function. It is clear from studies of infraprimate species, and studies of primate luteal cells, or luteinised granulosa cells, *in vitro*, that many molecules have the potential to affect steroid secretion. Clearly, the molecules with stimulatory or inhibitory effects *in vitro* which can be localised to the corpus luteum are all potential local regulators of steroidogenesis. However, the physiological role of these molecules remains unclear.

The IGFs have a probable role in folliculogenesis and it is likely that they have a local role in the corpus luteum. Work in the primate corpus luteum is limited, but IGFs, IGF receptors and IGFBPs can be localised to the corpus luteum of infraprimate species (Amselgruber *et al.*, 1994; Perks and Wathes, 1996; Juengel *et al.*, 1997). Indeed, there is increasing data that these molecules, in particular IGF-1 and IGFBP-3 can also be localised in the primate corpus luteum

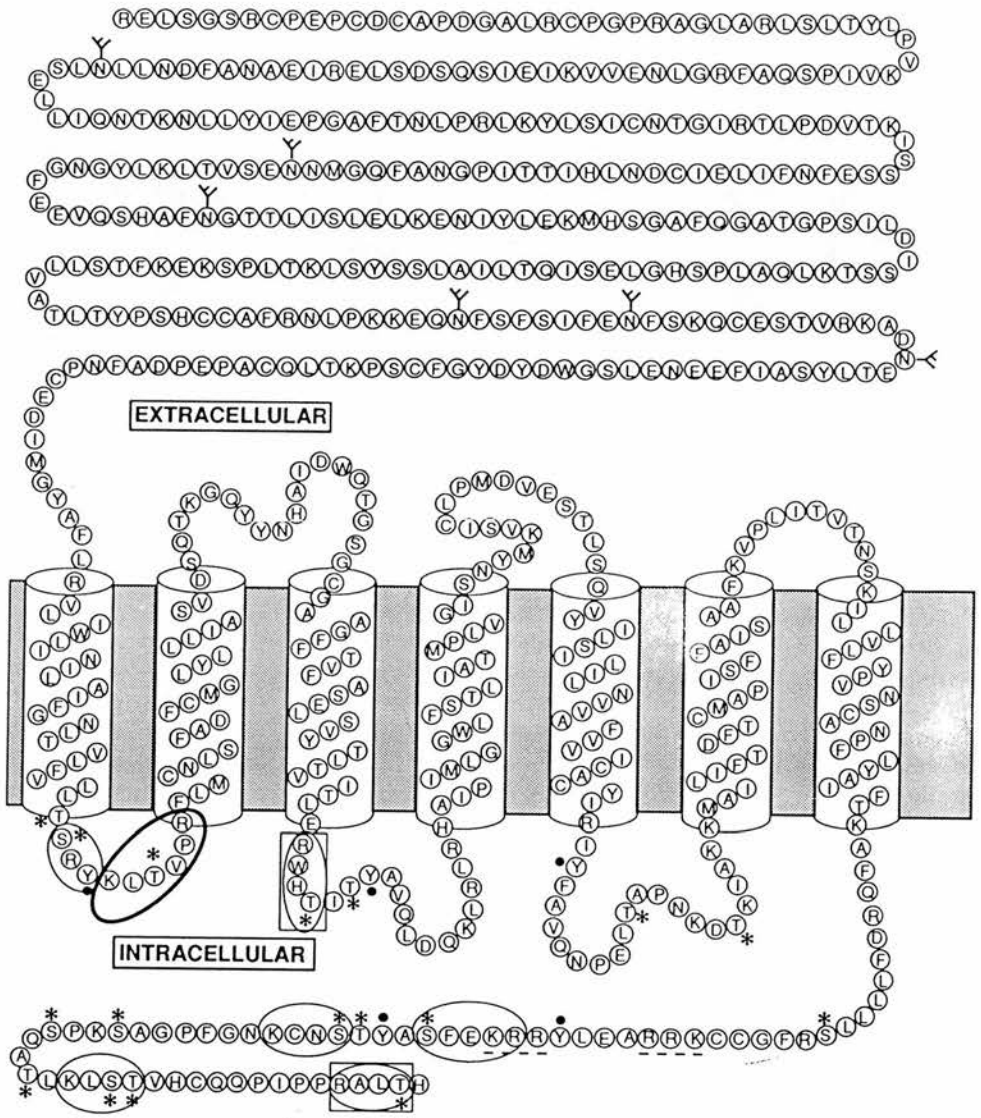

Figure 1.6

The structure of the LH receptor

Cartoon of the LH receptor showing the large extracellular N-terminal domain, with glycosylation points, the smaller intracellular C-terminal domain and the seven transmembrane loops.

(Segaloff and Ascoli, 1993)

(Hernandez *et al.*, 1992; Johnson *et al.*, 1996; Vandessel *et al.*, 1996). IGF-1 stimulates progesterone production from isolated luteal cells (Devoto *et al.*, 1995) and this effect seems to be mediated by specific IGF-1 receptors. IGF-1 receptors have been identified in human corpora lutea from spontaneous and clomiphene induced cycles (Hernandez *et al.*, 1992; Obasiolu *et al.*, 1992; Johnson *et al.*, 1996). IGFBP-3 localises to endothelial cells and may be involved in the local availability and actions of IGFs (Fraser *et al.*, 1997). Clearly the study of the role of IGFs in the primate corpus luteum is nascent.

Inhibins and activins may have a local paracrine effect in the corpus luteum. As discussed above, these molecules have been shown to have direct effects on steroidogenesis (Hillier *et al.*, 1991a). It is not clear, however, whether inhibin can affect luteal steroidogenesis *in vivo* or whether its anti-proliferative action (Matzuk *et al.*, 1992) is important in the corpus luteum. There is more evidence that activin inhibits luteal cell steroidogenesis (Hillier *et al.*, 1991a; Miro and Hillier, 1992; Brannian *et al.*, 1992). Activin binding sites, presumably activin receptors, can be detected within the corpus luteum (Woodruff *et al.*, 1993) and the subunits responsible for its production are clearly present (Fraser *et al.*, 1993; Roberts *et al.*, 1993). It is not known how important the inhibitory effect of activin is in the corpus luteum, but, in view of the recent data suggesting increased activin concentrations during luteolysis (Muttukrishna *et al.*, 1996), its role may be significant, and more work is clearly required.

Several authors have postulated a role for progesterone in controlling its own synthesis (Duffy *et al.*, 1994; Rothchild, 1996). Recent studies have shown that the human (Iwai *et al.*, 1990; Suzuki *et al.*, 1994) and non-human primate corpus luteum contains specific nuclear hormone receptors for progesterone (Hild-Petito *et al.*, 1988; Hild-Petito and Fazleabas, 1997). Progesterone receptor protein can be detected in the corpus luteum by immunohistochemistry (Hild-Petito *et al.*, 1988), western blotting (Duffy *et al.*, 1997), ligand binding (Slayden *et al.*, 1994) and mRNA can be detected by reverse transcriptase (RT) polymerase chain reaction (PCR) (Chandrasekher *et al.*, 1994). Co-localisation immuno-histochemical studies have suggested that the steroidogenic cells responsible for progesterone synthesis express genomic receptors for the progesterone they produce (Hild-Petito *et al.*, 1988; Suzuki *et al.*, 1994). In addition, some novel data using ovine and porcine corpora lutea have suggested that the corpus luteum has novel membrane associated progesterone binding proteins and these may be

non-genomic progesterone receptors (Bramley and Menzies, 1988; Menzies and Bramley, 1994; Bramley and Menzies, 1994).

Although the hypothesis of progesterone controlling its own production is attractive (Rothchild, 1981; Duffy *et al.*, 1994; Rothchild, 1996), its paracrine role in the corpus luteum is not clear. The progesterone receptor antagonist mifepristone (RU486) can induce luteolysis when administered in the luteal phase. However, RU486 affects luteotrophic gonadotrophin secretion (Schaison *et al.*, 1985; Batista *et al.*, 1994), and when gonadotrophins are given in combination with RU486 there is no effect (Croxatto *et al.*, 1989). Elegant studies investigating the effect of trilostane, an inhibitor of 3β-HSD, on luteal lifespan are now producing interesting results (Duffy *et al.*, 1994; Duffy and Stouffer, 1995; Duffy *et al.*, 1996). Although these studies have postulated several roles for progesterone in the local control of luteal function (Duffy *et al.*, 1994; Duffy and Stouffer, 1995), specific physiological roles for local progesterone have not yet been clearly identified.

Androgen receptors have been identified in primate corpora lutea (Suzuki *et al.*, 1994). They appear to be maximal just after ovulation (Horie *et al.*, 1992). Although androgens have been implicated in some forms of follicular atresia, their local role in the corpus luteum is not known. A direct action of androgens in the corpus luteum has not been demonstrated, but androgens inhibit progesterone synthesis and LH receptor expression on luteinised granulosa cells (Polan *et al.*, 1986). In addition, they can act with LH to stimulate inhibin production from luteinised granulosa cells (Hillier *et al.*, 1991b). Androgens have some anabolic effects. They stimulate cellular growth and may be involved in ECM deposition (Behrman *et al.*, 1993). However, at present their role in the corpus luteum is not clear.

Other molecules that can be detected in corpora lutea have been shown to affect steroidogenesis *in vitro* (Nappi *et al.*, 1994). Prostaglandin (PG) $F_{2\alpha}$ has been shown to have negative effects on steroidogenesis under a variety of conditions (Jordan, 1981; Dennefors *et al.*, 1982; Auletta and Flint, 1988; Abayasekara *et al.*, 1993). Other prostaglandins, notably PGE_2, have been shown to have pro-steroidogenic effects in some experiments (Dennefors *et al.*, 1982; Hahlin *et al.*, 1988; Zelinski-Wooten and Stouffer, 1990). This has lead several authors to postulate a role for the ratio of different prostaglandins in determining luteal function (Mitchell *et al.*, 1991; Olofsson and Leung, 1994). In addition,

arachidonic acid itself, and leukotrienes, have also been shown to have negative effects on steroidogenesis (Yoshimura *et al.*, 1992; Ciereszko *et al.*, 1995). Receptors for prostaglandins are present in primate corpora lutea (Powell *et al.*, 1974; Rao *et al.*, 1977a), and it is likely that they have a significant role in luteal function. The precise nature of that role, however, remains obscure (Olofsson and Leung, 1994).

Cytokines have clear effects on luteal function *in vitro* (Brännström and Norman, 1993; Nappi *et al.*, 1994). IFN-γ has been shown to inhibit basal, as well as hCG-stimulated, progesterone synthesis in human luteal cells (Wang HZ *et al.*, 1992). Various reports have suggested that IL-1, IL-2 and IL-6 have both pro- and anti-steroidogenic effects (Wang *et al.*, 1991; Sjögren *et al.*, 1991; Fukouka *et al.*, 1992; Brännström and Norman, 1993). One of the best studied cytokines in luteal function is TNFα. TNFα can be detected in corpora lutea (Roby and Terranova, 1989), particularly during luteal regression (Bagavandoss *et al.*, 1990). It has clear anti-steroidogenic effects in culture conditions (Wang HZ *et al.*, 1992; Fairchild Benyo and Pate, 1992). It is likely that cytokines are important in luteal function, but it remains to be shown whether their effects on steroidogenesis occur at physiological, rather than pharmacological, levels.

In summary, luteal steroidogenesis is absolutely dependent on LH from the pituitary gland. LH binds to LH receptors to activate intracellular messenger cascades, notably cAMP. Cyclic AMP stimulates the synthesis of StAR, the transport of cholesterol to the inner mitochondrial membrane, and the activity of the steroidogenic enzymes. This results in progesterone synthesis and secretion. Progesterone synthesis can be modified by various molecules acting in autocrine and paracrine pathways. These molecules include proteins, growth factors, cytokines, eicosanoids and steroids. These molecules are likely to exert a major influence on the control of luteal steroidogenesis. However, the exact role of these factors in luteal steroidogenesis *in vivo* is not yet clear.

1.6 Luteolysis

In the absence of conception, the corpus luteum will lose its functional and structural integrity. Historically, corpus luteum regression, or luteolysis, has been divided into two distinct stages: functional and structural luteolysis. Functional

luteolysis refers to the increasing failure of progesterone synthesis and secretion (Zeleznik and Fairchild Benyo, 1994), whereas structural luteolysis refers to the processes involved in the dissolution and resorption of the gland (Behrman *et al.*, 1993), that are classically thought to follow functional luteolysis (Luck and Zhao, 1995). These definitions were based on studies of the rat corpus luteum (Malven, 1969), where these processes are clearly separate events, under separate endocrine control (Niswender and Nett, 1994). In the human, there appears to be no clear temporal and endocrinological distinction between these stages of luteolysis (Behrman *et al.*, 1993). However, for the purpose of this discussion, the cessation of progesterone production can be viewed as one end-point of luteolysis and luteal involution as another.

1.6.1 The Cessation of Luteal Steroidogenesis

Progesterone synthesis and secretion by the corpus luteum is dependant on the action of the trophic hormone, LH, from the pituitary gland. One could postulate that changes in LH secretion during the luteal phase could be responsible for the initiation of luteal regression. Withdrawal of LH using gonadotrophin releasing hormone (GnRH) antagonists ($GnRH_{ant}$) during the luteal phase, results in a rapid loss of both the functional and structural integrity of the primate corpus luteum (Hutchison and Zeleznik, 1984, Fraser *et al.*, 1986). In fact, pituitary LH secretion does change as the luteal phase progresses. There is a progesterone-mediated reduction of the frequency of LH pulses from approximately one pulse per hour, during the early to mid-luteal phase, to one pulse every four to six hours, from the mid-luteal to the late-luteal phase (Ellinwood *et al.*, 1984). Some widely-read undergraduate textbooks state that luteolysis is secondary to the reduction of LH secretion, secondary to progesterone feedback on the hypothalamic-pituitary axis (O'Riordan *et al.*, 1982).

Luteolysis is not caused by alterations in pituitary LH pulse frequency. Maintenance of menstrual cycles in anovulatory rhesus monkeys and humans by unvarying GnRH pulse frequencies, typical of the follicular and early luteal phases, does not prolong the life span of the corpus luteum (Crowley and McArthur, 1980). Moreover, using a pulse frequency of one pulse per eight hours, typical of the late-luteal phase, throughout the luteal phase does not alter its duration (Hutchison *et al.*, 1986). When plasma concentrations of LH are reduced by 50% to 75%, by limiting the amount of GnRH delivered per pulse,

progesterone secretion is maintained (Zeleznik and Little-Ihrig, 1990). These findings suggest that during the early and mid-luteal phases there is a surfeit of LH. During the mid- to late-luteal phases the corpus luteum appears to become much less sensitive to the ambient concentrations of LH.

Although luteolysis cannot be attributed to hypothalamic pituitary regulation, it is possible that it results from the inability of LH to bind to the corpus luteum. In ruminants, there is a decline in both LH receptors and progesterone as luteolysis approaches (Guy *et al.*, 1995; Smith *et al.*, 1996a). However, it appears that the fall in progesterone production precedes the loss of LH receptors. A similar conclusion has been reached in non-human primates (Cameron and Stouffer, 1982). In addition, the binding affinity of the luteal LH receptor does not appear to consistently change throughout the luteal phase (Rao *et al.*, 1977b; Cameron and Stouffer, 1982; Ottobre *et al.*, 1984; Yeko *et al.*, 1989). It appears that falling progesterone production in spontaneous luteolysis is not preceded by a change in the number or affinity of the luteal LH receptors.

The presence of LH receptors *per se* is not the only dictate of receptor function in the corpus luteum. Coupling of these receptors to adenylyl cyclase and the generation of cAMP is vital for mediation of the action of LH (Segoloff and Ascoli, 1993). There is some evidence that LH receptors can uncouple from adenylyl cyclase, and that adenylyl cyclase itself may be regulated independently from the LH receptor (Eyster *et al.*, 1985; Rojas *et al.*, 1989). However, most data on LH receptors are from rodents or rodent cell lines (Segaloff and Ascoli, 1993). There is some indication that LH receptors behave differently in the corpus luteum of rodents and humans. Ligand causes LH receptor down regulation in rat corpora lutea (Peegel *et al.*, 1994), but does not appear to in the primate corpus luteum (Ottobre and Stouffer, 1986). Clearly, further work on the human luteal LH receptor is required.

It is not known whether failure of progesterone production is secondary to alterations in the levels of the steroidogenic enzymes. Steroidogenic enzymes are not expressed in the non-functioning corpus luteum at the time of menstruation (Doody *et al.*, 1990; Bassett *et al.*, 1991). Various studies have suggested a reduction of mRNA, and possibly protein, of the steroidogenic enzymes $P450_{scc}$ and 3β-HSD during the primate luteal phase (Bassett *et al.*, 1991; Suzuki *et al.*, 1993; Sanders and Stouffer, 1997). However, it is not clear whether this effect occurs before or after the fall in progesterone begins. Although StAR can be

detected in the human corpus luteum (Kiriakidou *et al.*, 1996), and it is the best candidate for the rate-limiting step of luteal steroidogenesis (Stocco and Clark, 1996), it is not clear how the expression of StAR changes throughout the functional luteal phase, or whether it is regulated independently from the other steroidogenic enzymes. The role of an alteration in steroidogenic enzyme expression during the functional lifespan of the corpus luteum is not clear.

1.6.2 The Involution of the Corpus Luteum

During the mid-luteal phase, at the peak of its functional activity, in a non-conception cycle, the corpus luteum is clearly visible on the surface of the ovary. During the early follicular phase of the next cycle, it is difficult to identify that corpus luteum on the ovary macroscopically (Zeleznik and Fairchild Benyo, 1994). Microscopically, it has become a small, relatively avascular, fibrous remnant (Corner, 1956). During this marked luteal involution, there has been a loss of luteal cells and a clear change in its size and structure. This involves marked remodelling of the ECM and the influx of fibroblasts (Adams and Hertig, 1969).

1.6.3 Apoptosis

There is good evidence that the loss of cells from the corpus luteum during luteolysis is by a form of physiological cell death known as apoptosis (Wyllie *et al.*, 1980; Arends and Wyllie, 1991). Indeed, apoptosis was first described in hormonally responsive tissues after loss of trophic support (Kerr *et al.*, 1972). Apoptosis is an active process involving the activation and expression of several different genes (Arends and Wyllie, 1991). The biochemical and morphological characteristics of apoptosis are now well established.

Cells undergoing apoptosis shrink and become denser with pyknotic nuclei. Their chromatin is cleaved, and the nucleus and cytoplasm break up into small membrane-bound packages called apoptotic bodies, that are phagocytosed by macrophages and neighbouring cells. These morphological changes of apoptosis can be seen in tissues by conventional light microscopy (Wyllie, 1994). However, apoptosis occurs rapidly, and apoptotic bodies are only visible for short periods of time in tissues. When they are easily seen, there tends to be high rates of apoptotic cell death within the tissue (Arends and Wyllie, 1991).

Biochemically, apoptosis is associated with a characteristic pattern of deoxyribonucleic acid (DNA) degradation. The DNA is cleaved between histones resulting in fragments which are multiples of 180-200 base pairs (bp). This gives rise to a typical laddering pattern when the DNA is separated by gel electrophoresis (Wyllie, 1994). In addition, techniques have now been developed to allow the *in situ* detection of DNA fragments. These *in situ* end-labelling techniques are claimed to detect apoptotic cells in tissue sections (Shikone *et al.*, 1996; Young *et al.*, 1997).

The early morphological studies of the corpus luteum described features which are now recognised as characteristic of apoptotic cell death (Corner, 1956). More recent studies, looking specifically at apoptosis, have shown morphological evidence of apoptosis in the corpora lutea of domestic animals and primates (Zheng *et al.*, 1994; Fraser *et al.*, 1995b). Apoptosis can also be detected by DNA laddering and *in situ* 3' end-labelling of oligonucleosomes in primate and ruminant corpora lutea (Juengel *et al.*, 1993; Rueda *et al.*, 1995a; Shikone *et al.*, 1996; Young *et al.*, 1997). There seems to be no doubt that apoptosis occurs in the corpus luteum.

If apoptosis is involved in the involution of the corpus luteum, the rates of apoptosis should increase during luteolysis and reduce during luteal 'rescue'. This appears to be what happens. Induction of luteolysis in the primate and ruminant clearly induces apoptosis (Juengel *et al.*, 1993; Fraser *et al.*, 1995b; Rueda *et al.*, 1995a; Young *et al.*, 1997). In women, regressing corpora lutea have the biochemical features of apoptosis, and these are not evident in the mid-luteal corpus luteum (Shikone *et al.*, 1996; Yuan and Giudice, 1997). Rates of apoptosis have been reported to be reduced in the corpus luteum of early pregnancy (Shikone *et al.*, 1996). In addition, studies *in vitro* have suggested a role for hCG in promoting luteal cell survival (Dharmarajan *et al.*, 1994). The loss of the corpus luteum from the ovary is likely to involve apoptotic cell death and luteal 'rescue' appears to inhibit apoptotic cell death.

Apoptosis is regulated by a variety of proteins, genes and proto-oncogenes (Hale *et al.*, 1996). Apoptosis and proto-oncogenes, particularly *bax* and *bcl-2* have clear roles in follicular atresia (Hsueh *et al.*, 1994; Tilly, 1996). These proteins can be detected in human corpora lutea (Rodger *et al.*, 1995; Rodger *et al.*, 1998), but they do not seem to change during the luteal phase or after luteal 'rescue' with hCG. Another proto-oncogene, *c-myc*, can also be detected in primate corpora

lutea (Fraser *et al.*, 1995b), and may be involved in induced luteolysis. However, at present, there is little convincing data about the role of proto-oncogenes in the apoptosis associated with luteolysis, and further work needs to be done.

1.6.4 Remodelling of the Extracellular Matrix

Remodelling is a term used to describe changes in size, shape and cellular composition during growth, development and atrophy of tissues. The corpus luteum undergoes extensive tissue remodelling throughout its functional lifespan. The elegant observational studies of Corner systematically documented the marked tissue changes in the corpus luteum throughout the luteal phase (Corner, 1956). As well as changes in the appearance and composition of the cellular component, he documented marked alterations in the supporting connective tissue. Connective tissue forms a large proportion of each tissue and it is primarily responsible for the shape and volume of different organs. It is composed of stromal elements, such as cellular basement membranes, vessels, carrying blood and lymph, and cells such as fibroblasts and macrophages. All these elements are found within a supporting ECM. One of the major features of tissue remodelling is the continued synthesis and breakdown of the ECM (Luck and Zhao, 1995). The degradation and synthesis of ECM is orchestrated by a variety of hormones, cytokines and local growth factors (Hulboy *et al.*, 1997). The enzymes responsible for this remodelling, however, are tightly controlled at a cellular level.

1.6.5 The Matrix Metalloproteinases

The MMPs are the key enzymes involved in the degradation of ECM proteins during tissue remodelling (Birkendal-Hansen, 1995; Hulboy *et al.*, 1997). They are the only secreted enzymes capable of denaturing fibrillar collagens, and are localised to areas of active tissue remodelling (Hulboy *et al.*, 1997). The MMPs are a group of structurally related, zinc-dependant, proteolytic enzymes which are tightly controlled, but active under physiological conditions (Birkendal-Hansen, 1995). At present, 17 different MMPs have been described (Hulboy *et al.*, 1997). Although most are secreted into the ECM (Matrisian, 1990), the most recently described MMPs are transmembrane enzymes expressed on cellular membranes (Hulboy *et al.*, 1997). Of the 17 MMPs, the ones that have been studied most are interstitial collagenase (MMP-1), gelatinase A (MMP-2) and gelatinase B (MMP-

9) (Woessner, 1991). These are the enzymes which are capable of degrading the major structural collagens, gelatines and elastins.

As the MMPs are highly active proteolytic enzymes, their activity is tightly controlled at several levels. The first level of control is the transcriptional activation of mRNA synthesis (Birkendal-Hansen, 1994). The second level of control is their synthesis as pro-enzymes which require proteolytic cleavage to be fully active (Woessner, 1991; Hulboy *et al.*, 1997). The third level of control is the presence of specific tissue inhibitors. Tissue inhibitors of metalloproteinases (TIMPs) bind to, and inhibit, MMP enzymes with a one-to-one stoichiometry (Matrisian, 1990). At present there are four members of the TIMP family. TIMP-1 and TIMP-2 are secreted proteins which are widely expressed (Hulboy *et al.*, 1997). TIMP-3 is found is association with the basement membrane, but is less well characterised, and TIMP-4 has recently been reported, but has not yet been fully investigated (Hulboy *et al.*, 1997). TIMPs are frequently regulated in co-ordination with MMPs (Salamonsen, 1996; Hulboy *et al.*, 1997).

Alterations in MMP activity have been shown to be important in several physiological and pathological processes. MMPs have been implicated in the spread of neoplastic tissues (Naylor *et al.*, 1994), as well as in remodelling of reproductive tissues, such as the endometrium at menstruation (Hulboy *et al.*, 1997), and the follicle wall at ovulation (Curry *et al.*, 1992). It is likely that MMPs are involved in luteinisation and luteolysis but, although they can be detected in infraprimate corpora lutea (Endo *et al.*, 1993a; Tsang *et al.*, 1995; Nothnick *et al.*, 1996), little is known about their expression and activity in the corpus luteum. A great deal more is known about the expression of TIMPs in the corpus luteum.

The corpus luteum expresses large amounts of TIMPs, indeed TIMP-1 has been shown to be one of the major secretory products of the ovine corpus luteum (Smith *et al.*, 1993). Although first described in the sheep corpus luteum, it is now clear that TIMP-1 is produced in large amounts by the corpus luteum of many species, including the rat (Nothnick *et al.*, 1995), sheep (Smith GW *et al.*, 1994), cow (Juengel *et al.*, 1994) and pig (Smith MF *et al.*, 1994). It is not just TIMP-1 that is expressed in the corpus luteum, TIMP-2 is also produced by corpora lutea of rats (Nothnick *et al.*, 1995), sheep (Smith *et al.*, 1995a) and cows (Smith *et al.*, 1996b), and that TIMP-3 can also be detected in rat ovaries (Nothnick *et al.*, 1995). It is likely that TIMPs have significant roles in the control of luteal structure and function.

The expression of TIMP-1 has been shown to change during $PGF_{2\alpha}$-induced luteolysis (Juengel *et al.*, 1994). In addition, exposure to gonadotrophins, during the LH surge, up-regulates TIMP-1 expression in preovulatory granulosa cells (Smith GW *et al.*, 1994). These observations support a hypothesis that the remodelling associated with luteolysis is modulated by a reduction in TIMP-1 expression and luteal 'rescue' by hCG is associated with an up-regulation in the expression of TIMP-1, or other TIMPs. This hypothesis implies a role for MMP activity during the functional lifespan of the corpus luteum. However, at present little is known about MMP expression during the lifespan of the corpus luteum, and nothing is known about their expression in the primate corpus luteum. In addition, it is not clear how MMPs can function in an environment which contains, by all accounts, a large excess of specific inhibitors.

To recap, in a non-conception cycle, the corpus luteum undergoes luteolysis. There is a reduction of LH-stimulated progesterone production. The molecular mechanisms for this decrease are not clear but are likely to involve an increasing block to steroidogenesis somewhere in the progesterone production pathway. In addition, the corpus luteum regresses structurally. This regression is likely to involve apoptotic cell death and tissue remodelling involving the MMPs. However, the molecular mechanisms, and controls, of these processes, particularly in the primate, are still unknown.

1.7 The Search for a Luteolysin

What makes the corpus luteum undergo luteolysis? The answer to that question is unknown, undoubtedly complex, and continues to baffle. Several molecules have been studied as potential luteolysins. Most of these molecules have been shown to have roles in infraprimate species. It is unlikely, however, that a single luteolysin exists in the primate. Clearly though, transducer molecules, other than LH, must be involved in the control of both functional and structural luteolysis in women. Identification of these molecules has proven difficult. However, the molecules implicated in luteolysis in other species may well have roles in the primate, to a greater or lesser extent (Auletta and Flint, 1988). These molecules are now discussed in detail and their relevance to primate physiology reviewed.

1.7.1 Oestrogen

The primate corpus luteum synthesises and secretes oestrogen (Lenton and Woodward, 1988; Sanders and Stouffer, 1997). Oestrogen is clearly luteolytic in some infraprimate species such as rabbits (Niswender and Nett, 1994). It has been suggested that locally produced oestrogens may facilitate the corpus luteum to self-destruct and lead to regression (Knobil, 1973). Direct injection of oestrogen into the ovary containing the corpus luteum results in premature menstruation (Gore *et al.*, 1973; Karsch and Sutton, 1976). This seems to be a local effect, as systemic oestrogen, or injection into the contralateral ovary did not shorten the menstrual cycle (Karsch *et al.*, 1973), and it, at that time, appeared that plasma LH concentrations were not significantly reduced (Karsch and Sutton, 1976). It has also been reported that the concentrations of oestrogens in the macaque corpus luteum increase during the late-luteal phase as the progesterone concentrations decline (Butler *et al.*, 1975). Therefore, under experimental situations oestrogens can indeed be luteolytic.

The physiological relevance of oestrogen-induced luteal regression however is not clear. Recent studies have failed to detect oestrogen receptors, by immunohistochemistry and RT-PCR analysis, in primate corpora lutea (Chandrasekher *et al.*, 1994). However, in the rabbit corpus luteum, where oestrogen is thought to be of physiological relevance, oestrogen receptors have been clearly identified (Lee *et al.*, 1971). The negative effects of oestrogen on the primate corpus luteum are now thought likely to be due to a suppressive effect on LH secretion (Schoonmaker *et al.*, 1982). Oestrogen does not induce regression when LH is added back (Hutchison *et al.*, 1987). It is likely that oestrogen-induced luteal regression is due to the suppression of LH secretion, and there is no evidence of a physiological role *in vivo*.

1.7.2 Prostaglandins

In non-primate mammals, luteolysis has been attributed to the uterine synthesis and secretion of $PGF_{2\alpha}$ (Niswender and Nett, 1994), and its subsequent transport to the ovary via a local countercurrent mechanism (Auletta and Flint, 1988). This is clearly not the case in primates, as hysterectomy does not affect the ovarian cycle, or the luteal life-span (Neill *et al.*, 1969a). Although non-primate mammals and primates have evolved alternative mechanisms of luteolysis, it is possible that they share some common effector molecules. There is good evidence to suggest

that $PGF_{2\alpha}$ is also luteolytic in non-human primates and women (Auletta *et al.*, 1984a; Auletta *et al.*, 1990).

Systemic $PGF_{2\alpha}$ is luteolytic when administered to smaller primates, such as the marmoset monkey, in pharmacological doses (Fraser *et al.*, 1995b). It is also luteolytic when administered to rhesus monkeys in the early stages of pregnancy (Wilks, 1980). Although pharmacological doses of $PGF_{2\alpha}$ do not alter cycle length in most primates (Wilks, 1980; Auletta and Flint, 1988), transient reductions in circulating progesterone have been reported after systemic, intra-luteal, and intra-arterial administration of $PGF_{2\alpha}$, in rhesus monkeys (Auletta *et al.*, 1973; Auletta and Flint, 1988) and women (Wentz and Jones, 1973; Auletta and Flint, 1988). There is a rapid metabolism of prostaglandins during their passage through the pulmonary circulation, that seriously interferes with their bioavailability (Samuelsson *et al.*, 1971). When $PGF_{2\alpha}$ is infused, at physiological rates, through a local mechanism, directly into the rhesus monkey corpus luteum, it has been shown to be luteolytic (Auletta *et al.*, 1984a).

If $PGF_{2\alpha}$ is a physiological luteolysin in non-human primates and women, it would need to be generated within the ovary or the corpus luteum itself (Auletta and Flint, 1988). Evidence for the local production of $PGF_{2\alpha}$ is however still contradictory. Some groups report higher concentrations of $PGF_{2\alpha}$ in human luteal tissue during luteolysis (Shutt *et al.*, 1976; Patwardhan and Lanthier, 1980), but other groups have failed to confirm this (Challis *et al.*, 1976; Swanston *et al.*, 1977). Measurement of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ ($DHKF_{2\alpha}$), the major circulating metabolite of $PGF_{2\alpha}$, in peripheral plasma, failed to show any change at the time of luteolysis (Auletta *et al.*, 1984b). However, the primate corpus luteum has the capacity to synthesise $PGF_{2\alpha}$ (Challis *et al.*, 1976; Balmaceda *et al.*, 1979), and $DHKF_{2\alpha}$ levels are higher in ovarian venous blood from the ovary containing the corpus luteum, when compared to the other ovary, during the late-luteal phase in the rhesus monkey (Auletta *et al.*, 1984b). In addition, like non-primate mammals (Niswender and Nett, 1994), the corpus luteum of non-human primates and women express $PGF_{2\alpha}$ receptors (Powell *et al.*, 1974; Rao *et al.*, 1977a).

$PGF_{2\alpha}$ is not the only prostaglandin which has been shown to affect luteal function. *In vitro*, whereas $PGF_{2\alpha}$ inhibits luteal function (Auletta and Flint, 1988), PGE_2, PGD_2 and PGI_2 generally stimulate luteal function (Zelinski-Wooten and Stouffer, 1990; Zeleznik and Fairchild Benyo, 1994). Injections of

indomethacin, a cyclooxygenase (COX) inhibitor, into rhesus monkeys, at doses that prevented the initiation of labour, failed to affect the life-span of the corpus luteum (Manaugh and Novy, 1976). However, infusion of the prostaglandin synthase inhibitor, meclofenamate, into the corpus luteum of the rhesus monkey, during the mid-luteal phase, actually caused premature luteolysis (Sargent *et al.*, 1988). This suggests that prostaglandin synthesis may be required for normal luteal function at this time. Indeed, it has been demonstrated that direct infusion of PGE_2 into the corpus luteum inhibits $PGF_{2\alpha}$-induced luteal regression in monkeys (Zelinski-Wooten and Stouffer, 1990). However exogenous PGE_2 infusion, during the time of luteal regression, did not extend the life-span of the corpus luteum. If prostaglandins are involved in luteal function, their net effect is likely to depend on the relative abundance of luteotrophic and luteolytic forms (Olofsson and Leung, 1994).

1.7.3 Oxytocin

Exogenous oxytocin has been shown to induce luteal regression or insufficiency in ruminants (Auletta and Flint, 1988; Niswender and Nett, 1994). Although these effects were initially considered pharmacological, several lines of evidence suggest that oxytocin may have a physiological role during luteolysis in ruminants. It has become clear that the mechanism of action of oxytocin is to stimulate uterine secretion of the luteolytic agent $PGF_{2\alpha}$ (Auletta and Flint, 1988). Immunisation against endogenous oxytocin was found to delay luteolysis (Sheldrick *et al.*, 1980). It has also become clear that oxytocin is synthesised, stored and released from the steroidogenic cells of the ruminant corpus luteum (Wathes and Swann, 1982). At the time of luteolysis, there is an increase in the sensitivity of the uterus to oxytocin, and $PGF_{2\alpha}$ secretion increases (Roberts *et al.*, 1976; Sheldrick and Flint, 1985). It is therefore likely that luteal oxytocin is involved in luteolysis at a systemic, rather than a local, level.

Some *in vitro* data suggests, however, that oxytocin may have direct effects on luteal steroidogenesis. Oxytocin has an inhibitory effect on hCG-stimulated progesterone synthesis in dispersed bovine luteal cells (Tan *et al.*, 1982a; Tan *et al.*, 1982b), although this finding is not universal (Rodgers *et al.*, 1985). Oxytocin receptors can, however, be detected on luteal cells (Niswender *et al.*, 1985), although whether these are steroidogenic or endothelial is not clear (Auletta and Flint, 1988). Indeed, it is possible that oxytocin has direct effects on luteal blood

flow, as it has been shown that oxytocin alters the blood flow in many organs (Auletta and Flint, 1988).

The role of oxytocin in the primate is less clear. As the ovarian cycle and luteolysis continues in the absence of a uterus (Neill *et al.*, 1969a), oxytocin cannot have the same role. However, oxytocin receptors are present in the primate corpus luteum (Khan-Dawood *et al.*, 1993) and oxytocin is synthesised in the primate corpus luteum (Einspanier *et al.*, 1994; Dawood and Khan-Dawood, 1986). Although some authors have reported some inhibitory effects of oxytocin on stimulated progesterone production by isolated human luteal cells (Tan *et al.*, 1982a; Bennegard *et al.*, 1987), this has not been found by others (Richardson and Masson, 1985). However, oxytocin in pharmacological, rather than physiological, doses does seem to have some luteolytic effects in monkeys (Auletta *et al.*, 1984c). The role of oxytocin in primate luteal steroidogenesis and local blood flow remains to be clarified.

1.7.4 Prolactin

In the rat, prolactin serves an essential role in the formation and maintenance of the corpus luteum (Niswender and Nett, 1994). While $PGF_{2\alpha}$ induces functional luteolysis, it does not directly cause cellular degeneration in rat or bovine luteal cells (Jordan, 1981). In the rat, prolactin is the agent that induces structural involution of the corpus luteum (Endo *et al.*, 1993a). Paradoxically, prolactin is luteotrophic in the rat during luteinisation (Niswender and Nett, 1994). Differentiation of functional corpora lutea is induced by prolactin secretion in response to vaginal and cervical stimulation during mating (Spies and Niswender, 1971). Prolactin appears to work by inducing the expression of LH receptors in the corpus luteum following their down-regulation at ovulation (Richards and Williams, 1976). However, after functional luteolysis has occurred, prolactin induces structural luteolysis. The mechanisms of action of prolactin in the rat corpus luteum are not clear. As prior exposure to indomethacin will block the luteolytic effect of prolactin (Sanchez-Criado *et al.*, 1987), it appears that prior exposure to prostaglandins is necessary. $PGF_{2\alpha}$ may sensitise the luteal cells to the luteolytic effects of prolactin.

It is unlikely that prolactin has similar luteotrophic and luteolytic actions in the primate. However, prolactin receptors have been described in the human ovary and corpus luteum (McNeilly *et al.*, 1980; Bramley *et al.*, 1987). It has been

reported that inhibition of prolactin secretion using bromocriptine, a dopaminergic agent, will slightly shorten the luteal phase, and reduce the peak progesterone levels, when given to women with normal menstrual cycles (Schulz *et al.*, 1978). On the other hand, short luteal phases have also been reported associated with hyperprolactinaemia (Doody and Carr, 1991). It is clear, however, that prolactin can interfere with pituitary gonadotrophin production (Bohnet and McNeilly, 1979), and this may explain its effects on luteal function. Indeed, although one study has shown a direct effect of prolactin on luteal cell steroidogenesis *in vitro* (Hunter, 1984), most studies find that exogenous prolactin does not appear to influence progesterone production from dispersed luteal cells (Stouffer *et al.*, 1980; Tan and Biggs, 1983). At present, there does not appear to be a distinct role for prolactin in the control of the primate corpus luteum.

1.7.5 Immune Cells and Cytokines

Immune cells and their cytokine products appear to be normal constituents of the ovary and corpus luteum (Brännström and Norman, 1993). It is possible that they have essential roles in the regulation of ovarian function. There are several pieces of evidence which point to local interactions between the immune and reproductive systems. White blood cells have clearly been shown to be involved in the ovulatory process (Brännström and Norman, 1993). Interfering with the immune system in mice can cause failure of proper ovarian development (Nishizuka and Sakakura, 1969). Glucocorticoids, which block leukocyte influx and impair immune cell cytokine production, prevent luteal regression in the rat (Wang *et al.*, 1993). In addition, the induction of lymphopaenia has been shown to cause luteal dysfunction in the cow (Alila and Hansel, 1984). Indeed, luteolysis is associated with a leukocyte infiltration which has been documented in rodents (Brännström *et al.*, 1994b), rabbits (Naftalin *et al.*, 1997), ruminants (Murdoch, 1987; Lei *et al.*, 1991) and women (Wang LJ *et al.*, 1992; Brännström *et al.*, 1994a).

Immune cells have the potential to directly affect steroidogenesis. The presence of macrophages increases progesterone synthesis in cultures of murine and human luteinised granulosa cells (Kirsch *et al.*, 1981; Halme *et al.*, 1985). The addition of leukocytes, or leukocyte conditioned medium, has the same effect (Emi *et al.*, 1991). Although, these reports suggest a role for immune cells in the promotion of steroidogenesis, it is increasingly clear that there is an influx of these cells during

luteolysis (Brännström and Norman, 1993). It is therefore likely that products of these cells, that have a negative effect on steroidogenesis, are important during luteolysis.

Several immune cell products have been shown to be negative regulators of steroidogenesis. Leukocytes are prominent generators of prostaglandins (*vide supra*) and reactive oxygen species (*vide infra*). The best studied cytokine, TNFα, is a product of macrophages that is released in luteal tissues in response to macrophage activation (Bagavandoss *et al.*, 1990). It can be detected in the bovine, human and rat corpus luteum (Roby and Terranova, 1989; Brännström and Norman, 1993). TNFα appears to exert some anti-steroidogenic effects on cultured human granulosa cells (Fukuoka *et al.*, 1992). It acts with other cytokines to augment their inhibition (Wang HZ *et al.*, 1992) of progesterone production. In addition, TNFα is anti-steroidogenic to bovine and pig luteal cells (Veldhuis *et al.*, 1991; Fairchild Benyo and Pate, 1992), where it also stimulates the synthesis of $PGF_{2\alpha}$ (Fairchild Benyo and Pate, 1992). Indeed, a TNFα-like factor has been shown to increase during luteolysis in the ewe (Ji *et al.*, 1991) and cause luteolysis when infused into pig corpus luteum (Wuttke *et al.*, 1993). Specific TNFα receptors have also been described in pig corpora lutea (Richards and Almond, 1994). Therefore immune cell TNFα is a good candidate for one of the tissue mediators of luteolysis.

1.7.6 Reactive Oxygen Species

Reactive oxygen species are generated in a variety of biological conditions and are known to inhibit cellular synthetic and metabolic pathways. The cytochrome P450 enzymes, involved in steroidogenesis, appear to be particularly sensitive to inhibition by free oxygen radicals (Behrman and Preston, 1989; Endo *et al.*, 1993b). Therefore, these highly reactive molecules, such as the superoxide anion (O_2•-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•), are clear candidates for effector molecules during luteolysis (Behrman *et al.*, 1993). There is now increasing evidence that reactive oxygen species mediate some of the early events during luteolysis in the rat, and possibly the primate, corpus luteum.

Reactive oxygen species are generated in the rat corpus luteum in response to exogenous $PGF_{2\alpha}$ and during natural luteal regression (Riley and Behrman, 1991). Exposure of luteal cells to H_2O_2 results in marked inhibition of steroidogenesis (Behrman and Preston, 1989). Although the cells remain viable, they display a

rapid inhibition of LH-dependent cAMP and progesterone production (Margolin *et al.*, 1990). Generation of superoxide radicals in luteal cells in response to $PGF_{2\alpha}$ precedes the decline in progesterone production (Sawada and Carlson, 1991). It is therefore likely that part of the mechanism of action of $PGF_{2\alpha}$ on luteal cells is through the generation of reactive oxygen species.

Reactive oxygen species have multiple sites of action on the steroidogenic pathway. They rapidly inhibit LH-induced cAMP synthesis (Behrman and Preston, 1989). This effect appears to be independent of the numbers and affinity of LH receptors, or the activity of adenylyl cyclase (Behrman and Preston, 1989). It has been suggested that there is a functional uncoupling of LH receptors from adenylyl cyclase (Segaloff and Ascoli, 1993). This is rapid, and appears to be related to a decrease in membrane fluidity, secondary to membrane lipid peroxidation (Riley and Carlson, 1987). In addition, H_2O_2 blocks cAMP-dependent steroidogenesis. This effect does not seem to be related to intracellular mobilisation of cholesterol or inhibition of $P450_{scc}$ activity (Behrman and Aten, 1991). It appears to be at the level of protein synthesis (Musicki *et al.*, 1993), inhibiting the translocation of cholesterol to the inner mitochondrial membrane (Behrman and Aten, 1991). It seems that inhibition of StAR synthesis is one of the anti-steroidogenic effects of reactive oxygen species.

It is still uncertain if reactive oxygen species are involved in luteolysis in the primate, where the physiological relevance of $PGF_{2\alpha}$ as a luteolysin is unclear. *In vitro*, H_2O_2 has luteolytic-like effects on granulosa-lutein cells (Endo *et al.*, 1993b). The effects on the steroidogenic pathway appear to be similar to those observed in rat luteal cells (Musicki *et al.*, 1993). In addition, there appears to be a direct inhibition of the activity of the steroidogenic enzymes $P450_{scc}$, 3β-HSD and $P450_{arom}$ (Behrman and Preston, 1989; Endo *et al.*, 1993b). Although it is not known if H_2O_2 is generated in the human corpus luteum *in vivo*, there is some evidence of its production *in vitro* (Michael *et al.*, 1994). It remains possible that reactive oxygen species serve a functional role in mediating luteal regression in women.

1.8.7 Regulation of Blood Supply

Early observational studies of the ovine corpus luteum during luteolysis reported marked early blanching, and decrease in luteal blood flow (Niswender *et al.*, 1976). As the blood supply is important in bringing nutrients and substrates to

luteal cells, regulation of the local vasculature may have a role in the luteolytic process. Prostaglandins themselves have marked effects on blood flow (Nett *et al.*, 1976), and they are clearly involved in regulation of blood vessel tone during menstruation (Abel, 1985). In addition, the corpus luteum contains various vasoactive substances including nitric oxide (NO) (Shiels *et al.*, 1996), endothelins (Flores *et al.*, 1995), and vasoactive cytokines (Brännström and Norman, 1993). Effects on the vasculature, including apoptosis of endothelial cells, have been reported during induced and natural luteolysis, in the primate and bovine corpus luteum (Fraser *et al.*, 1995c; Modlich *et al.*, 1996).

In sheep, serum progesterone is closely correlated to the rate of blood flow to the ovary containing the corpus luteum (Niswender *et al.*, 1976). Indeed, in this species, decreased luteal flow is associated with an inadequate luteal phase (Niswender and Nett, 1994). This correlation is not only seen in sheep, it exists to a degree in women. Blood flow to the corpus luteum has been examined by colour doppler ultrasound studies. Maximum blood flow was seen in the mid-luteal phase, a time of maximal progesterone production (Tinkanen, 1994; Bourne *et al.*, 1996). There was a positive correlation between the systolic blood flow velocity, luteal volume and serum progesterone in women. Low luteal blood flow rates have been shown to have an association with inadequate progesterone production, and miscarriage (Salim *et al.*, 1994; Glock and Brumsted, 1995; Kupesic and Kurjak, 1997).

The role of luteal blood flow in the corpus luteum in relation to its function is not clear. It is not known whether the decline in luteal flow is a cause or effect of declining progesterone secretion, or indeed, if both are secondary to a another unknown process. Steroid hormones have vasoactive properties, and hCG itself may have effects of blood vessel tone (Toth *et al.*, 1994). It is not clear if changes in luteal blood flow are involved in the luteolytic process, but the early evidence suggests they may be associated with it. However, the regulation of luteal blood flow and local factors involved in its control are not known. Clearly, this is an area requiring further study.

In summary, the effector molecules involved in stimulating the processes involved in luteolysis are not clear in the human. There is evidence that $PGF_{2\alpha}$, cytokines, especially TNFα, reactive oxygen species and vasoactive molecules may be involved. However, their role, and control at a molecular level, remain to be determined. The factors responsible for their production during luteolysis, and

how this is prevented by hCG during luteal 'rescue', are still not clear. However, the next topic to be considered is the process of luteal 'rescue' during maternal recognition of pregnancy.

1.8 Maternal Recognition of Pregnancy

In the absence of a successful pregnancy, the corpus luteum has a finite life span. Luteal progesterone production starts declining from the mid-luteal phase onwards and will cease after an average of 14 days (Lenton and Woodward, 1988). However, if conception occurs, progesterone production from the corpus luteum is maintained (Stouffer, 1988), and is increasing at the time when luteolysis normally would have occurred (Figure 1.7) (Lenton and Woodward, 1988; Tovanabutra *et al.*, 1993). These findings led to the conclusion that the corpus luteum is "rescued" during early pregnancy (Neill *et al.*, 1969b).

Mechanisms of maternal recognition of pregnancy differ between species. Clearly, that of the primate is of fundamental importance for the purpose of this thesis. However, it is wise to review the disparate mechanisms involved in maternal recognition of pregnancy in infraprimate species. Often an understanding of these mechanisms will put the situation in the primate into context, and give pointers to potential additional effector molecules in the primate.

1.8.1 Infraprimate Species

Progesterone must be secreted throughout pregnancy in most mammalian species. This provides a stable uterine environment to nurture the fetus throughout gestation. In some species, such as carnivores and marsupials, gestation lasts no longer than a functional luteal phase (Niswender and Nett, 1994). In these species, there is no need for the conceptus to alter luteal function to support its gestation. Most species, however, have evolved mechanisms to shorten the luteal phase to allow earlier return to fertility in the event of a non-conception cycle. The luteal phase is lengthened in a conception cycle. This is achieved by the conceptus-uterine unit being involved in the inhibition of luteolysis throughout gestation, or until the feto-placental unit can produce enough progesterone of its own to maintain pregnancy.

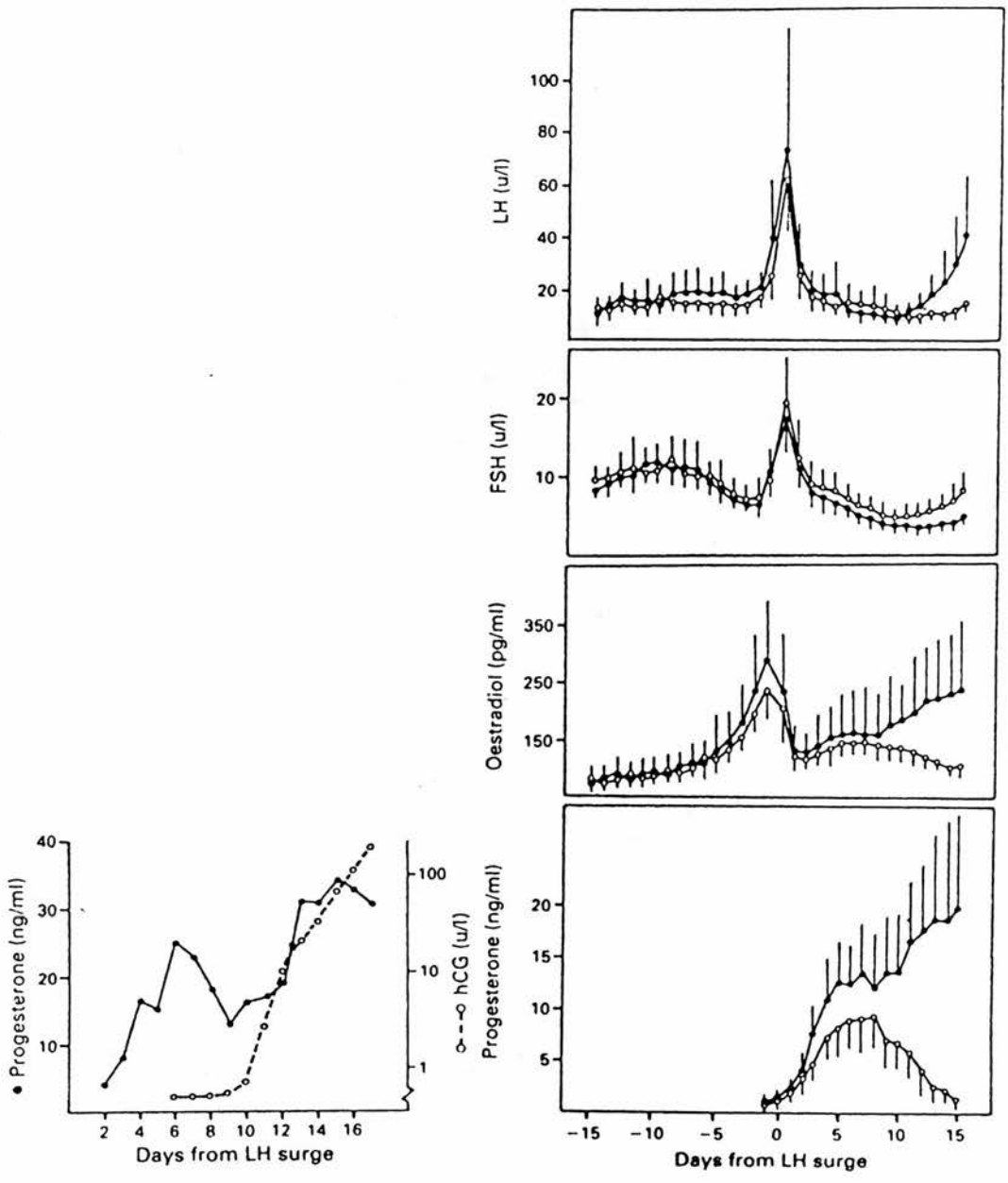

Figure 1.7 Daily geometric mean (and 68% confidence limits) of LH, FSH, oestradiol and progesterone concentrations in 27 non-conception cycles (○—○) and 26 conception cycles (●—●).

Endocrinology of a conception cycle

Representative graph of the plasma progesterone concentrations in women throughout the luteal phase in a conception and a non-conception cycle. In a conception cycle, the increasing progesterone concentrations are maintained through the late-luteal phase. The implanting blastocyst secretes hCG in logarithmically increasing concentrations.

(Lenton and Woodward, 1988)

1.8.2 Rodents

The corpus luteum of the rat is very transient indeed (Smith *et al.*, 1975). However, the act of cervical stimulation during mating, or experimentally, prolongs the lifespan of the corpus luteum for up to 12 days (Spies and Niswender, 1971). A cervical neuroendocrine feedback reflex causes prolactin concentrations to rise. This prolactin acts on the corpus luteum to maintain and enhance progesterone output. Indeed, daily injections of prolactin will maintain luteal function in an unstimulated female (von Berswoldt-Wallabre *et al.*, 1964), and an inhibitor of prolactin terminates pseudopregnancy (Gibori *et al.*, 1974).

The gestation of a rat is 22 days however, and ovarian progesterone is required throughout most of this period (Niswender and Nett, 1994). Further prolongation of the lifespan of the corpus luteum during actual pregnancy is therefore required. Maintenance of the corpus luteum during the second half of gestation in rats depends on the secretion of placental luteotrophins (Niswender and Nett, 1994). These appear to be a mixture of placental lactogen, a prolactin-like molecule, and a type of chorionic gonadotrophin, an LH-like molecule (Gibori *et al.*, 1974; Jayatilak *et al.*, 1984). These have direct stimulatory effects on luteal progesterone secretion and reduce the sensitivity of the corpus luteum to the luteolytic effects of the uterine $PGF_{2\alpha}$ (Niswender and Nett, 1994), that continues to be secreted throughout gestation.

1.8.3 Rabbits

Rabbits are stimulated to ovulate by mating (Niswender and Nett, 1994) and their corpus luteum secretes progesterone for 16 to 17 days. The trophic hormone for luteal function is oestradiol produced by LH action on ovarian follicles (Bill and Keyes, 1983). In the absence of pregnancy, the corpus luteum undergoes luteolysis, probably as a result of uterine $PGF_{2\alpha}$ secretion. Indeed, it may be the increasing concentrations of oestradiol which stimulate its secretion (Browning *et al.*, 1980). During pregnancy, the corpus luteum is maintained for the 31 days of gestation. A placental product is responsible for this prolongation, but this factor does not have gonadotrophin-like properties (Niswender and Nett, 1994). It is likely that the presence of the conceptus prevents the secretion of luteolytic $PGF_{2\alpha}$ in response to increasing oestradiol. Although not fully characterised, it may be that the placental luteotrophic factor is PGE_2, which is secreted in high concentrations by the rabbit conceptus (Lytton and Poyser, 1982).

1.8.4 Guinea Pigs

In the guinea pig, uterine $PGF_{2\alpha}$ is responsible for luteolysis after 11 to 13 days (Niswender and Nett, 1994). Placental progesterone production maintains pregnancy, but the contribution of the corpus luteum is required throughout the first 30 days of gestation. The function of the corpus luteum must therefore be prolonged by the feto-placental unit in pregnancy. The exact mechanisms whereby this is achieved are not fully clear, but it is likely to be a combination of a reduction in uterine $PGF_{2\alpha}$ secretion, and the secretion of a placental chorionic gonadotrophin with LH-like properties (Babra *et al.*, 1984).

1.8.5 Ruminants

In sheep and cows, luteolysis is attributed to the uterine synthesis and secretion of $PGF_{2\alpha}$, and its subsequent transport to the ovary via a local countercurrent mechanism (Auletta and Flint, 1988). In the ewe, the corpus luteum must be present through the first 50, and in the cow the first 200, days of gestation (Niswender and Nett, 1994). The conceptus must prevent normal luteal regression. This is achieved probably by a combination of inhibiting uterine $PGF_{2\alpha}$ production and reducing the sensitivity of the corpus luteum to $PGF_{2\alpha}$. (Niswender and Nett, 1994). It is now generally accepted that the conceptus in sheep and cattle secrete a trophoblastic protein (TP) (ovine TP (oTP-1) and bovine TP (bTP-1)) which inhibits pulsatile secretion of $PGF_{2\alpha}$ from the pregnant uterus. There is considerable homology between the structures of oTP-1 and IFN-α (Imakawa *et al.*, 1989), and both molecules have antiviral activity (Poniter *et al.*, 1988). Secretion of uterine $PGF_{2\alpha}$, and its metabolites, around the time of luteolysis is inhibited during pregnancy. However, basal $PGF_{2\alpha}$ levels are higher in the pregnant state than the non-pregnant state (Niswender *et al.*, 1994). This implies a reduced sensitivity of the corpus luteum to $PGF_{2\alpha}$.

The luteotrophic effect of the embryo is exerted locally. Anastomosis of the uterine vein draining the gravid horn to the opposite uterine vein results in maintenance of the corpus luteum on the opposite ovary (Niswender and Nett, 1994). Intrafollicular or arterial injections of $PGF_{2\alpha}$ are less efficient in inducing luteal regression in pregnancy (Auletta and Flint, 1988), confirming that the corpus luteum is made more resistant to the luteolytic effects of $PGF_{2\alpha}$. This effect is likely to be mediated by a conceptal product as, in the presence of two embryos, the corpus luteum is more resistant (Niswender and Nett, 1994). This

antiluteolytic signal may be PGE_2. Conceptuses secrete PGE_2, and infusion of PGE_2 into the uterine lumen prolongs luteal lifespan for the duration of the infusion (Shemesh *et al.*, 1979; Reynolds *et al.*, 1983). It is possible that other embryonic proteins are involved but these remain to be characterised.

1.8.6 Pigs

The corpus luteum must continue to secrete progesterone throughout gestation in the pig (Niswender and Nett, 1994). In pigs, luteolysis is associated with an increased uterine secretion of $PGF_{2\alpha}$. Porcine conceptuses secrete oestrogen and PGE_2. Infusion of oestrogen maintains the corpus luteum (Frank *et al.*, 1977). It is not fully characterised but it is likely that local oestrogens and PGE_2 are involved in maternal recognition of pregnancy in the sow.

1.8.7 Horses

Maternal recognition of pregnancy in the horse is not well understood. It is likely that uterine $PGF_{2\alpha}$ is responsible for luteal regression and that the conceptus prevents its action (Niswender and Nett, 1994). There is a clear reduction in uterine $PGF_{2\alpha}$ secretion during early pregnancy (Douglas and Ginther, 1976). There is a need for interaction with the endometrium and the conceptus interacts with as much lining endometrium as possible (Leith and Ginther, 1984). The substance involved in reducing $PGF_{2\alpha}$ secretion may be oestrogen. The conceptus synthesises oestrogen *in vitro*, and exogenous oestrogen has these effects *in vivo* (Niswender and Nett, 1994). This delays luteolysis until about day 35 when the conceptus produces large amounts of equine chorionic gonadotrophin (eCG). Follicles present on the ovaries at this time ovulate and produce secondary corpora lutea (Niswender and Nett, 1994). The primary and secondary corpora lutea are stimulated to produce progesterone until the feto-placental unit takes over, at about 150 days of gestation. At this time the eCG disappears from the circulation. Progestational support for the remainder of the pregnancy is provided by the feto-placental unit.

1.8.8 Primates

It is clear from infraprimate species that, commonly, luteotrophic substances are secreted by the uterine-conceptus unit. These substances prolong the luteal lifespan. The same is true of the primate. In 1927 some elegant experiments

demonstrated that urine from pregnant women stimulated luteinisation in rats (Zeleznik and Fairchild Benyo, 1994). This was the first demonstration of a specific luteotrophic factor of pregnancy. It became clear that this substance was separate from the gonadotrophins found in menopausal women's urine, as it did not possess the ability to stimulate follicular growth. This gonadotrophin was not of pituitary origin, as media from cultured trophoblast contained this luteotrophic factor in large amounts (Gey *et al.*, 1938). Further experiments showed that injections of this chorionic gonadotrophin into rhesus monkeys, during the luteal phase, prolonged the functional lifespan of the corpus luteum (Zeleznik and Fairchild Benyo, 1994). The conceptus is therefore the source of the luteotrophic signal which 'rescues' the corpus luteum. That luteotrophic signal is hCG.

The structure and functional characteristics of hCG have now been well documented (Iles and Chard, 1993). It is a glycoprotein hormone with structural similarities to LH, FSH and thyroid stimulating hormone (TSH). Like these other hormones it consists of two subunits: a common α-subunit linked to a specific β-subunit. The β-subunit of hCG is similar to that of LH, but with differences in size and glycosylation pattern (Iles and Chard, 1993). The hCG β-subunit shares the amino acid sequence with LH β-subunit, but has an additional 30 amino-acids at the carboxy-terminal (Pierce and Parsons, 1981). Gene analysis suggests that the gene for hCG β-subunit is derived from that of the LH β-subunit, but the stop codon has been altered to lengthen the molecule (Iles and Chard, 1993). The additional amino acids, and thus the different glycosylation pattern, make hCG and LH have different properties in the circulation. The plasma half-life of hCG is substantially longer than that of LH (36 hours vs. approximately 20 minutes) (Rizkallah *et al.*, 1969; Morell *et al.*, 1971).

The lifespan of the corpus luteum is extended in early pregnancy by hCG secreted by the developing syncytiotrophoblast. In women, hCG can first be detected in the circulation nine to ten days after the LH surge (Lenton and Woodward, 1988). This rises logarithmically to peak levels at eight to twelve weeks of gestation, and then declines to lower, but detectable, levels throughout the remainder of pregnancy (Zeleznik and Fairchild Benyo, 1994). The role, if any, of hCG after the luteo-placental shift is not known. Although hCG can first be detected in the peripheral circulation ten days after the LH surge, there is evidence for production of hCG by the preimplantation blastocyst (Fishel *et al.*, 1984), and hCG can be detected in concentrated urine extracts before its appearance in the plasma (Baird

et al., 1991). It is likely that hCG begins to exert its effect before it can be detected in the peripheral plasma by conventional methods.

It has clearly been shown in both the non-human primate (Stouffer *et al.*, 1987) and women (Illingworth *et al.*, 1990) that exogenous logarithmically increasing hCG can 'rescue' the corpus luteum, and mimic the hormonal changes of early pregnancy. Clearly at this stage of luteal 'rescue', hCG alone is enough to maintain normal luteal function. However, maintenance of the corpus luteum with exogenous hCG for more than 10 days has not been possible in the primate model (Stouffer *et al.*, 1987). At this stage, luteal function is still required for the maintenance of pregnancy (Csapo *et al.*, 1973). Although it is possible that additional factors from the conceptus or uterus are necessary for prolonged luteal function, it is likely that the doses of hCG in these experiments were inadequate, or the exposure *in vivo* was not physiological. It seems highly likely that hCG is the sole molecule required for luteal 'rescue'.

Some investigators claim that other products from the conceptus or the uterine-conceptus unit, in addition to hCG, may be important during luteal 'rescue' (Johnson *et al.*, 1993; Lower *et al.*, 1993). The basis of their argument is that progesterone levels are lower in ectopic gestations, at a stage when hCG concentrations are the same as intrauterine gestations. However, trophoblast function in ectopic pregnancy is impaired (Ledger *et al.*, 1994) and the corpus luteum has a dynamic response to hCG, in that increasing hCG concentrations are required to produce the same levels of progesterone, as the corpus luteum ages (Knobil, 1973; Lenton and Woodward, 1988; Tovanabutra *et al.*, 1993). It is likely that the explanation for their observations (Johnson *et al.*, 1993; Lower *et al.*, 1993) is the dynamic response of the corpus luteum to a suboptimal rate of rise in hCG concentrations. To date, no other endocrine factors have been shown to have a role in luteal 'rescue'.

How does hCG achieve luteal 'rescue'? It is clear that hCG acts through the LH receptor (Cole *et al.*, 1973; Segaloff and Ascoli, 1993). Stimulation of this receptor on the steroidogenic cells maintains steroidogenesis. Both exogenous LH and hCG can stimulate luteal cell progesterone synthesis *in vivo* and prolong the luteal phase (Hanson *et al.*, 1971). However, logarithmically increasing concentrations of hCG are required to maintain progesterone synthesis (Stouffer *et al.*, 1987; Stouffer, 1988, Lenton and Woodward, 1988). This implies that the steroidogenic cells are becoming increasingly insensitive to the trophic effects of

hCG. However, the molecular mechanisms of this effect are obscure. In addition, hCG must prevent apoptosis (Dharmarajan *et al.*, 1994) and tissue remodelling. As LH receptors are found on steroidogenic cells (Nishimori *et al.*, 1995), cell products from these cells must influence the rest of the gland during 'rescue'. The molecular nature of how structural luteolysis is prevented by hCG, like functional luteolysis, remains unknown. Clearly, further studies are required.

The continued function of the corpus luteum in early pregnancy is essential until its endocrine activities, notably progesterone production, are assumed by the placenta (Stouffer *et al.*, 1987). Oophorectomy or luteectomy prior to the development of sufficient placental steroidogenesis causes abortion (Csapo *et al.*, 1973). However, luteectomy after six or seven weeks of gestation does not result in abortion (Csapo *et al.*, 1973). At this stage, the placenta is secreting enough progesterone to maintain pregnancy, and the corpus luteum is essentially functionally redundant. This transition is known as the luteo-placental shift after which serum hCG begins to decline (Hearn, 1986; Tovanabutra *et al.*, 1993).

1.8.9 The Corpus Luteum during Pregnancy

To recap, there is great variation within mammalian species of the role of the corpus luteum throughout gestation. In some species, the corpus luteum is present throughout gestation, and its presence is absolutely required for the maintenance of pregnancy (Niswender and Nett, 1994). In other species, the corpus luteum is required for varying percentages of the length of gestation (Niswender and Nett, 1994; Zeleznik and Fairchild Benyo, 1994). In the human, the corpus luteum is only required for maintenance of pregnancy during the first trimester of gestation. Thereafter, the progesterone required to maintain pregnancy is derived from the trophoblast cells of the maturing placenta. The stage of pregnancy when the placenta produces enough steroids to maintain pregnancy and the corpus luteum becomes functionally redundant, as described above, is known as the luteo-placental shift (Zeleznik and Fairchild Benyo, 1994).

Endocrinological studies have been designed to track the relative contributions of the corpus luteum and the placenta to circulating progesterone concentrations throughout gestation. This is possible because the corpus luteum contains the steroidogenic enzyme 17α-hydroxylase, that is not found in the placenta (Tulchinsky and Hobel, 1973). The corpus luteum therefore secretes some 17α-hydroxyprogesterone as well as progesterone, and these hormones have a similar

pattern of secretion (Adashi, 1994). Although the adrenal gland produces some 17α-hydroxyprogesterone, the vast majority in the blood throughout gestation is of luteal origin (Adashi, 1994).

Plasma 17α-hydroxyprogesterone concentrations peak on the fourth to fifth week of gestation. Thereafter there is a marked decline (Tulchinski and Hobel, 1973). Progesterone concentrations, however, continue to rise after the fifth week of gestation and therefore, from this stage onwards, the placenta is the major source of circulating progesterone (Knobil, 1973). Ovariectomy of rhesus monkeys at this stage does not result in a significant fall in serum progesterone concentrations (Knobil, 1973). Indeed, towards the end of the first trimester circulating hCG concentrations also decline (Hearn, 1986). It is likely that this occurs after the luteo-placental shift but the molecular basis of this decline is not fully understood. After five weeks of completed gestation, the placenta is the primary source of progesterone in the primate.

Although successful pregnancies occur if the ovaries are removed after the luteo-placental shift, the human corpus luteum does not become non-functional during the rest of gestation. The corpus luteum can sometimes be clearly identified on the ovary at the time of caesarean section at term (Emmi *et al.*, 1991). In addition, although the steroid output wanes, it does not disappear completely. At term, venous blood draining the ovary bearing the corpus luteum has greater concentrations of progesterone than the contralateral side (Emmi *et al.*, 1991). Indeed, human ovarian luteal relaxin secretion has been reported throughout gestation (Sherwood, 1994). The physiological significance of the primate corpus luteum throughout gestation, after the luteo-placental shift, is not known.

In summary, hCG rescues the corpus luteum and maintains its progesterone secretion for a further four weeks. Until this stage, the progesterone produced by the corpus luteum is absolutely required to support an early pregnancy. After this stage, the placenta provides enough progesterone to maintain the pregnancy and the corpus luteum becomes functionally redundant. The molecular mechanisms of how hCG prevents luteolysis are not known. However it is known that hCG acts through the LH receptor which is found on the cell membranes of luteal cells. The molecular control of luteolysis and luteal 'rescue' in the primate remain an enigma.

1.9 The Continuing Enigma of the Corpus Luteum

This chapter has served to review our current understanding of ovarian and luteal structure and function. It is clear that there are large deficiencies in our current knowledge of the corpus luteum. Our understanding of the molecular mechanisms of luteolysis and how they are prevented by hCG during maternal recognition of pregnancy in women is limited. One of the factors which has limited our knowledge of the human is the fact that infraprimate species control their corpus luteum in different ways.

1.9.1 Model systems

Although some parallels of luteal function can be seen between primates and infraprimate species, there are marked differences. This means that infraprimate species have only limited use as models for the human corpus luteum. It also means that data collected on infraprimate cannot be applied to the human corpus luteum without independent testing. It is easy to study laboratory and farmyard animals, under careful experimental conditions. It is much harder to study non-human primates and women. This has, therefore, limited the opportunities to investigate the human corpus luteum. It is clear that, to increase our knowledge of human luteal function, a primate model is required. The best primate model is, of course women. However, few groups throughout the world have access to human corpora lutea. When human corpora lutea are available, these tend to be archival samples or samples collected on an ad-hoc basis. This means that exact dating of the corpora lutea in relation to the LH surge is difficult.

1.9.2 Early Pregnancy

One of the problems in studying the human corpus luteum, and the transition between luteolysis and luteal 'rescue', is the lack of availability of suitable luteal tissue from early pregnancy. It is unethical to collect such tissue, as luteectomy in early pregnancy causes miscarriage. Some groups have obtained early pregnancy corpora lutea from ectopic pregnancies. However, these often present around the time of the luteo-placental shift, and when the corpus luteum is failing. Although these corpora lutea have proven useful, they are clearly less than ideal. It is possible to obtain corpora lutea from naturally pregnant monkeys, and this limited approach has been used. A more user-friendly technique, where monkey corpora

lutea are collected after luteolysis has been prevented by treatment with exogenous hCG, has also been utilised. However, no studies on early 'rescued' human corpora lutea have been possible. This has clearly been a bar to our understanding.

1.9.3 Luteolysis

There is no doubt that, in the primate, when compared to infraprimate species, luteolysis is more gradual. This is probably because primates do not utilise a systemic luteolysin. Although carefully timed primate corpora lutea, collected throughout the cycle,can give insights into natural luteolysis, the more gradual luteolytic process has the potential to mask some important changes. Therefore some investigators have used models of induced luteolysis in the primate to investigate specific changes. These approaches have not been used in women.

1.9.4 The Scope of this Thesis

This thesis reports work on the human corpus luteum. A model system has been developed to allow the collection of carefully dated corpora lutea from women throughout the functional luteal phase. Analysis of these corpora lutea has the potential to map the functional and structural changes during the normal luteal phase and during natural luteolysis. This model system has the potential to overcome a lot of the problems involved in studying the human corpus luteum.

In addition, the model system also allows for the collection of 'rescued' corpora lutea from simulated early pregnancy. Maternal recognition of pregnancy can be simulated using exogenous hCG to mimic the hormonal changes of early pregnancy. This means that, for the first time, the 'rescued' human corpus luteum can be studied and compared to corpora lutea from throughout the luteal phase, including those at the time of natural luteolysis.

In addition, an induced luteolysis model has also been developed. Unfortunately the drugs required to induce luteolysis have yet to be formally licensed for the treatment of women. This means that a non-human primate model has to be used. This has the benefits of being a primate model, involving co-ordinated luteolysis, and, because the ovaries are much smaller, allowing the examination of extra-luteal ovarian tissue. Together, these models have the potential to allow detailed investigation of the molecular mechanisms involved in controlling the luteolysis-luteal 'rescue' transition in the primate.

The experimental chapters of the thesis are laid out in two sections. Each section begins with a brief introduction to set the scene for the following chapters. The first section covers the functional aspects of the corpus luteum during luteolysis and simulated maternal recognition of pregnancy. The second section deals with the structural aspects of luteolysis and maternal recognition of pregnancy. At the end of the thesis, these sections are combined in a final discussion which tries to expand our understanding of the human corpus luteum, on the basis of the experimental work reported here. Firstly however, the materials and methods used in these studies are described in detail.

Chapter 2

Subjects and Tissue Collection

2.1 Experimental Models

There are three distinct stages during the normal functional life-span of the human corpus luteum: the early-luteal phase when the corpus luteum is forming and increasing its production of progesterone; the mid-luteal phase when the corpus luteum is fully formed and its progesterone production is maximal; the late-luteal phase when the progesterone production falls and the gland begins to regress. The fall in progesterone production by the corpus luteum in the late-luteal phase is prevented by exposure to logarithmically increasing concentrations of hCG from the trophoblast during maternal recognition of pregnancy. This 'rescued' corpus luteum can be considered a fourth stage of the luteal life-span.

In order to investigate the effects of luteal 'rescue' at a structural and functional level, a model has been designed to allow the collection of corpora lutea from all four of these luteal stages. Human corpora lutea were collected at the time of hysterectomy during carefully monitored menstrual cycles. They were dated on the basis of the urinary LH surge, in conjunction with the date of the last menstrual period (LMP), and the assessment of endometrial morphology. In addition, a protocol using injections of hCG was used to mimic the hormonal changes of early pregnancy, before some corpora lutea were collected. This regime 'rescues' the corpus luteum and allows the study of the molecular effects of maternal recognition of pregnancy.

The process of luteolysis is a gradual one in the primate, occurring over several days. The molecular changes of luteolysis may therefore be diluted over the course of the late-luteal phase. An additional model was therefore utilised to produce a rapid co-ordinated luteolysis. In the primate, luteolysis can be induced by acute LH withdrawal in the mid-luteal phase. This is achieved by systemic treatment with $GnRH_{ant}$. As this experimental treatment has not yet been licensed for use in women, a non-human primate model, using the marmoset monkey, was utilised. One benefit of this model is that luteolysis can also be induced by systemic treatment with $PGF_{2\alpha}$. It is therefore possible to compare and contrast

luteolysis induced indirectly, through withdrawal of trophic support, or directly by a known luteolysin.

These models allow the investigation of the structural and functional characteristics of the primate corpus luteum during the normal luteal phase, at the time of luteal 'rescue', during maternal recognition of pregnancy, and during co-ordinated luteolysis. Together they are unique and powerful tools for the investigation of the primate corpus luteum during the functional luteal phase.

2.2 Human Tissue

2.2.1 Recruitment of Patients

This study was approved by the Reproductive Medicine Sub-Committee of the South East Scotland Medical Ethics Committee. Informed consent was obtained from all women involved and their consultant gynaecologists. The case records of all women on the waiting list for hysterectomy, at the Royal Infirmary of Edinburgh, under the age of 45 years, were obtained and scrutinised at regular intervals. Healthy women, with regular menstrual cycles, who had not received any form of hormonal therapy in the last three months, who did not have a history of infertility, and who were having an open abdominal hysterectomy for benign conditions, were identified.

A letter (Fig. 2.1) was sent to these women approximately two months before the planned date of surgery. This letter briefly introduced the nature of the research and invited the women to contact a telephone extension with a 24-hour answering service if they were interested in participating. All the respondents were contacted by telephone. During this conversation the clinical details were confirmed, the study protocol was explained in detail, and any questions were answered.

If the woman was still interested in participating in the research, a detailed menstrual history was taken to determine how long after her expected menses the operation would take place. Each woman was classified on this basis as either 'unsuitable', 'routine' or 'suitable for rescue'. 'Unsuitable' women were those who did not fulfil the inclusion criteria after detailed discussion, or those whose operation would occur in the follicular phase of the cycle. 'Routine' women were those whose operation would fall in the luteal phase of the cycle. If the operation

MRC
Medical Research Council

MRC Reproductive Biology Unit
Centre for Reproductive Biology
37 Chalmers Street
Edinburgh EH3 9EW

telephone 031-229 2575
fax 031-228 5571

Your reference

Our reference
«Data P.Illingworth Let»

«date»

«name»
«street»
«district»
«city»
«postcode»

Dear «fullname»

I gather that you have a date to come into the Royal Infirmary for surgery and I am therefore writing to you to seek your help with some important research while you are in hospital. This research will investigate the establishment of early pregnancy in women. This is a very important area as problems such as infertility and early miscarriage are unfortunately very common. We are therefore trying to improve our knowledge of the vital process of pregnancy recognition in women so that we can better help such problems.

The research would basically involve having a small biopsy taken from one of your ovaries while you are asleep at the time of your operation. This will not add anything to the discomfort normally experienced at your operation and in addition there will be not effect on your future hormone production. We propose to use such tissue to study in great detail the processes by which the ovary recognises a pregnancy and thus produces sufficient hormone to maintain the pregnancy.

If you would like to know more about this research or think you may be willing to take part please telephone our office (031-229 2902 — 24 hour answering service) leaving your name and telephone number. I will then contact you to give you more information about the study and arrange a convenient time to discuss the project with you further. This does **not** commit you to taking part and you will be entirely free to withdraw at any time.

It is also, very important to emphasise that participation in this research is **entirely voluntary** and therefore if you are not interested in taking part please disregard this letter. Participation or otherwise in the research project will have no effect whatsoever on the treatment that you receive at the Royal Infirmary.

I would be most grateful for any help that you are able to offer.

Yours sincerely

Peter Illingworth
Clinical Consultant

Figure 2.1

Letter sent to suitable women

This letter was sent to all women identified on the elective waiting list for abdominal hysterectomy who fulfilled the inclusion criteria of the study. During the period 1993 to 1995 it was sent to 395 women, 45% of whom responded.

was expected to occur in the interval of two days before expected menses to two days after expected menses, these women were classified as 'suitable for rescue'. No further action was taken if the woman was classified as 'unsuitable'. Those classified as 'routine' or 'suitable for rescue' entered the pre-operative stage of the protocol.

2.2.2 Pre-operative Protocol

Specimen bottles and patient information sheets (Fig. 2.2) were given to all women classified as 'routine'. These women were instructed to collect an early morning specimen of urine each day, from 7 days after their LMP until hospital admission, and store these in their home freezer. On admission to hospital, these urines were collected and stored at -20 °C until analysis. On the day of surgery, a plasma sample was collected and this was stored at -20 °C until analysis. Plasma progesterone concentration was measured using an in-house progesterone radioimmunoassay (RIA) (Djahanbakhch *et al.* 1981a). This assay typically had a intra-assay coefficient of variation (CV) of <4%, a inter-assay CV of <11%, and a detection limit of 0.1 nmol/l.

Women classified as 'suitable for rescue' were visited at home and given a patient information sheet (Fig. 2.3). Those who did not consent to hCG administration were offered the opportunity to be treated as 'routine'. Those who agreed to participate were instructed to collect daily urine samples from day 7 of the menstrual cycle, and store these frozen at home. These samples were collected on day 18 or 19 of their menstrual cycle and assayed for LH concentration using an in-house assay (Djahanbackhch *et al.* 1981b). At 29 IU/l this assay typically has an intra-assay CV of 4% and an inter-assay CV of 10%. The date of ovulation was defined as the date of any clear single urinary LH peak after adjustment for urinary creatinine concentration. This date was classified as LH+0. Ovulation occurs within 36 hours of the start of the serum LH surge (Djahanbackhch *et al.* 1981b). It is generally accepted, however that ovulation occurs within 24 hours of the urinary LH surge (LH+1).

Daily intramuscular injections of hCG (Profasi; Serono, Welwyn Garden City, Herts, UK) were given from LH+7 until operation (5 to 8 days, mean 7.5 days). Dosage commenced at 125 IU (LH+7) and doubled daily, 250 IU (LH+8), 500 IU (LH+9), 1000 IU (LH+10), 2000 (LH+11), 4000 (LH+12), 8000 (LH+13) and 16 000 (LH+14). Plasma samples were taken on LH+7, LH+9, LH+11 and LH+13

THE MECHANISM OF PREGNANCY RECOGNITION IN WOMEN

PATIENT INFORMATION SHEET

CONTROL GROUP

Research into the establishment of early pregnancy in women.

What is the research about?

The establishment of early pregnancy is a very important process in women and problems such as infertility and early miscarriage are unfortunately very common. It is known that a woman's body will recognise pregnancy as a result of a hormone called hCG which is produced by the early embryo. This hormone hCG stimulates the ovary leading to all the other changes of pregnancy. However, we do still not understand the critical processes by which the ovary recognises this hCG and thus acts to maintain the pregnancy. This research is therefore intended to improve our understanding of early pregnancy.

What will be involved?

Each month at the time of a period, the ovary forms new cells which produce hormones and then release an egg at the time of ovulation. If pregnancy does not occur, these cells continue to produce hormone for 14 days before they die at the time of the next period. In this research we propose to remove these cells (which will die anyway) from the ovary at the time of your operation. This will extend the operation by approximately five minutes but will not add anything to the discomfort normally experienced at such an operation. In addition there will be no effect on your future hormone production.

In addition you will be asked to collect urine samples during the days leading up to your operation in order that we can measure your normal hormone production.

Your participation or otherwise in this research will not in anyway affect the treatment you will receive at the Royal Infirmary and you will be free to withdraw at any time.

Further information is available from Dr Colin Duncan, Clinical Research Fellow, MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh. Telephone 031 229 2575.

Figure 2.2

Patient information sheet for routine collection

It the patients were suitable for recruitment, they were given this information sheet and instructed to collect daily urine samples. Of all the women who collected urines, 65% had a suitable corpus luteum collected at surgery.

THE MECHANISM OF PREGNANCY RECOGNITION IN WOMEN

PATIENT INFORMATION SHEET

HCG TREATMENT GROUP

Research into the establishment of early pregnancy in women.

What is the research about?

The establishment of early pregnancy is a very important process in women and problems such as infertility and early miscarriage are unfortunately very common. It is known that a woman's body will recognise pregnancy as a result of a hormone called hCG which is produced by the early embryo. This hormone hCG stimulates the ovary leading to all the other changes of pregnancy. However, we do still not understand the critical processes by which the ovary recognises this hCG and thus acts to maintain the pregnancy. This research is therefore intended to improve our understanding of early pregnancy.

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Each month at the time of a period, the ovary forms new cells which produce hormones and then release an egg at the time of ovulation. If pregnancy does not occur, these cells continue to produce hormone for 14 days before they die at the time of the next period. In this research we propose to remove these cells (which will die anyway) from the ovary at the time of your operation. This will extend the operation by approximately five minutes but will not add anything to the discomfort normally experienced at such an operation. In addition there will be no effect on your future hormone production.

In order to investigate how the ovary responds to hCG, you will be given small amounts of hCG by daily injection for up to eight days. This is a naturally occurring female hormone which is in regular use in infertility treatment at much larger doses with no significant side-effects. In particular there will be no pregnancy symptoms at this very low dosage and no long term effects on your body.

Finally, blood samples will be required on alternate days in the days leading up to your operation and you will be asked to collect daily urine samples in order that we can measure your normal hormone production. Your participation or otherwise in this research is entirely voluntary and will not in anyway affect the treatment you will receive at the Royal Infirmary and you will be free to withdraw at any time. We will not be able to make any payments to volunteers for helping us with this research but we will meet the cost of any expenses incurred by volunteers.

Further information is available from Dr Colin Duncan, Clinical Research Fellow, MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh. Telephone 031 229 2575.

Figure 2.3

Patient information sheet for luteal 'rescue'

This information sheet was given to all women suitable for treatment with exogenous hCG to 'rescue' the corpus luteum prior to surgery. During the three years of the study satisfactorily 'rescued' corpora lutea were obtained from 12 women.

and stored at -20 °C until analysis. Concentrations of intact hCG were measured in these samples using an immunometric assay (hCG Serono MAIAclone; Intersep Ltd., Wokingham, Berks, UK). This method was calibrated against the First International Reference Preparation 75/537 and the inter-assay CV was typically 8%. Plasma hCG concentrations increased exponentially in these patients (Illingworth *et al.* 1990; Illingworth *et al.*, 1996). This regimen has previously been shown to mimic the hormonal changes of early pregnancy (Illingworth *et al.* 1990).

2.2.3 Collection of Tissue

Whole corpora lutea were enucleated from the ovary, at the time of hysterectomy, by blunt dissection, and the ovary was oversewn. Corpora lutea were collected at the beginning of the operative procedure before interruption of uterine or ovarian blood flow. The tissue was immediately divided into radial blocks in order to ensure that the whole thickness of the gland was represented in any piece. Two pieces of tissue were rapidly snap frozen in liquid nitrogen and stored at -70 °C for subsequent RNA and protein extraction. One piece was frozen in embedding medium (Tissue-Tek OCT compound; Miles Inc., Elkhart, IN, USA) and stored at -70 °C until frozen sections were cut. Another piece was fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin wax for subsequent histological, and immunohistochemical examination. In addition, an endometrial biopsy was obtained from the uterus immediately after removal. This was also fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin wax. During the course of the study, human placental tissue was required. This was obtained from the Simpson Memorial Maternity Pavilion which is connected to the research building. Placental tissue was either used fresh, or snap frozen and stored at -70 °C until required.

2.2.4 Dating of Corpora Lutea

LH concentrations were measured in each of the serial urine samples as described above. The LH surge was defined as the date of the peak urinary LH concentration, after standardisation for urinary creatinine concentration. If no LH peak, or more than one disparate peak, was detected in the serial urine samples, the associated corpus luteum was not classified, or used in any definitive studies. Corpora lutea were classified as early-luteal, if collected one to five days after the

urinary LH peak (LH+1 to LH+5). Likewise, they were classified as mid-luteal from LH+6 to LH+10, and late-luteal from LH+11 to LH+14. Those corpora lutea collected after hCG administration were classified as 'rescued'. In all cases, morphological dating of the luteal phase endometrium using the method of Li *et al.* (1988) was used to confirm the luteal phase classification. Only patients whose urinary LH dating, LMP, and endometrial dating were in agreement were used in subsequent definitive studies. Marked decidualisation of the endometrium was seen after seven days treatment with exogenous hCG.

2.2.5 Distribution of Corpora Lutea

During the three-year period (1993-1995), 598 sets of case records were scrutinised. Letters were sent out to the 395 (66%) women identified as suitable. Of these, 177 women replied, a response rate of 45%. Urine samples were collected by 90 (51%) of these women who were classified as 'routine' or 'suitable for rescue' prior to operation. A corpus luteum was obtained from 59 (65%) of these women. Of these corpora lutea, strictly accurate dating, by the above criteria, was not possible for 14. Therefore, 45 (76%) accurately dated corpora lutea were available for definitive study during this period. Of these, 10 (22%) were classified as early-luteal, 11 (24%) as mid-luteal, 12 (27%) as late-luteal and 12 (27%) as 'rescued'. Plasma progesterone concentrations were 30.6 ±8.4 nmol/l in the early-luteal phase, 38.6 ±7.2 nmol/l in the mid-luteal phase, 18.7 ±6.5 nmol/l in the late luteal phase and, after luteal 'rescue', progesterone concentrations increased from a mean of 37.8 ±5.6 nmol/l at the time of initiation of hCG treatment, to 52.5 ±0.9 nmol/l at the time of collection (Figure 2.4). One corpus luteum was obtained on average for every 13 sets of case notes analysed (7.5%).

2.2.6 Patient Characteristics

The mean age of the donors was 40 (range 29 to 42). The commonest indication for hysterectomy was menorrhagia (50%), followed by fibroids (25%) and pelvic pain, including endometriosis, (18%). At least one ovary was conserved at the time of operation in 82% of cases.

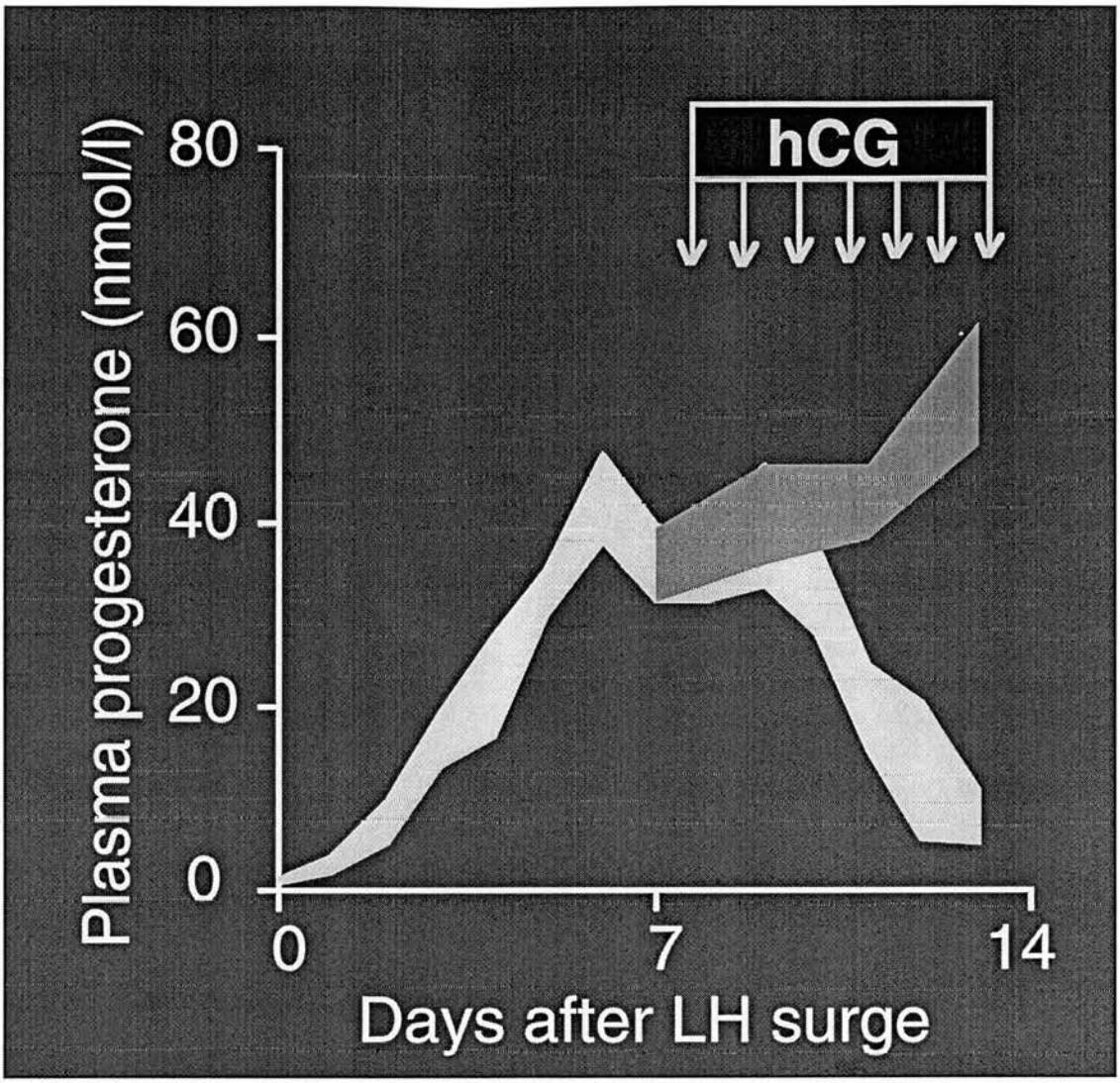


Figure 2.4

Progesterone concentrations during the luteal phase

Mean progesterone concentrations ± the standard error of the mean (S.E.M.) in female plasma at different stages of the luteal phase, and after luteal 'rescue' with logarithmically increasing exogenous hCG from LH+7. The stages of the luteal phase are also shown. Early = LH+1 to LH+5; Mid = LH+6 to LH+10; Late = LH+11 to LH+14. Resc = daily doubling hCG from LH+7 for 5 to 8 days. Progesterone concentrations increase in the early-luteal phase to a maximum in the mid-luteal phase and then decline in the late-luteal phase. After luteal 'rescue' progesterone concentrations are maintained and increase from LH+11 to LH+14.

(Groome *et al.*, 1996; Illingworth *et al.*, 1996)

2.3 Marmoset Tissue

2.3.1 Marmoset Colony

All experiments involving non-human primates were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Captive-bred common marmoset monkeys (*Callithrix jacchus jacchus*) were maintained in a colony which has been closed since 1973. Animals were housed in rooms that were maintained at temperatures between 20 °C and 25 °C and artificially lit between 07.00 h and 19.00 h. Animals were fed on 'Mazuri' New World Diet (Scientific Diet Services Ltd., Stepfield, Essex), fresh fruit, vegetables and seeds daily with water available *ad libitum*. Blood samples (300 µl) were collected on alternate days by femoral venepuncture without anaesthesia while the animals were held in a restraining device (Hearn *et al.*, 1978). These samples were assayed for progesterone to determine the date of ovulation and the luteal phase duration (Smith *et al.*, 1990). The marmoset monkey normally ovulates 2-3 follicles and has a functional luteal phase of approximately 18-21 days.

2.3.2 Treatment Protocols

Luteolysis was induced in the mid-luteal phase using either a $PGF_{2\alpha}$ analogue or $GnRH_{ant}$. Ovaries were collected on day 10 of the luteal phase from control animals or animals treated with either a 1 µg intramuscular (i.m.) injection of the $PGF_{2\alpha}$ analogue, cloprostenol (Planate; Coopers Animal Health Ltd., Crewe, Cheshire, UK), or a 500 µg/kg subcutaneous (s.c.) injection of the $GnRH_{ant}$, antarelix ([N-Ac-D-Nal^1, D-pCl-Phe^2, D-Pal^3, D-$(Hic)^6$, $Lys(iPr)^8$, D-Ala^{10}] GnRH; Europeptides (GEIE), Argenteuil, Val-D'Oise, France) (Deghenghi *et al.* 1993), 12 hours or 24 hours previously.

2.3.3 Collection of Tissue

The animals were sedated using 100 µl i.m. ketamine hydrochloride (Parke-Davis Veterinary, Pontypool, Gwent, UK) and killed with an intravenous (i.v.) injection of 400 µl sodium pentobarbitone (Euthetal; Rhône Mérieux, Harlow, Essex, UK). Whole ovaries were removed immediately, fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin wax. In addition, some marmoset ovaries were frozen in embedding medium (Tissue-Tek OCT compound) after removal. Frozen sections were prepared from these ovaries and stored at -70 °C until use.

A bank of normal marmoset tissues, including follicular phase ovaries, collected from this and other experiments, was also utilised. These tissues were removed immediately post-mortem. A piece of each tissue was snap frozen in liquid nitrogen and stored at -70 °C for subsequent RNA extraction. Another piece was frozen in embedding medium, and stored at -70 °C until frozen sections were prepared.

2.3.4 Distribution of Ovaries

Ovaries were collected from control animals, estimated to be day 10 of the luteal phase of the ovarian cycle. Some control animals had their ovaries frozen in embedding medium (n=4) and some had their ovaries fixed in paraformaldehyde and processed into paraffin wax (n=4). Ovaries frozen in embedding medium were also available from animals in the follicular phase of the cycle (n=4). Ovaries were also collected after systemic treatment to induce luteolysis. Ovaries were collected 12 hours after $PGF_{2\alpha}$ treatment and fixed in paraformaldehyde (n=3). Ovaries collected 24 hours after $PGF_{2\alpha}$ treatment were either fixed (n=3) or frozen (n=4). Ovaries collected 12 hours after $GnRH_{ant}$ treatment were fixed in some animals (n=3) and frozen in others (n=2). Similarly, ovaries collected 24 hours after $GnRH_{ant}$ treatment were either fixed (n=3) or frozen (n=4).

Chapter 3

General Materials and Methods

3.1 Source of Reagents

3.1.1 Source of Chemicals and Solutions

All reagents used were obtained from Sigma Chemical (Poole, Dorset, UK) unless otherwise indicated. Restriction enzymes and RNA polymerases were purchased from Promega (Southampton, Hants, UK). Iodinated LH (Chelsea Reagent) was obtained commercially from Department of Chemical Pathology, Hammersmith Hospital, London, UK. The specific activity of the iodinated LH is 100 μCi/μg and 10 000 counts per minute (c.p.m.) is equivalent to 45 pg. The reverse zymography kit was obtained commercially from University Technologies International Inc. (Calgary, Alberta, Canada). Ovine luteal cell conditioned medium, for use as a positive control for TIMP-1, was kindly provided by Prof M.F. Smith (University of Missouri, Columbia, USA).

3.1.2 Source of Antibodies

Prof M.R. Waterman of Vanderbilt University, Nashville, TN, USA provided the polyclonal rabbit antibody to human 17α-hydroxylase. The mouse monoclonal antibodies to human CD 68 (PG-M1) and von Willebrand factor were obtained commercially (Dako Ltd., High Wycombe, Bucks, UK). The polyclonal rabbit antibody to human placental type I 3β-HSD was kindly provided by Prof Van Luu-The (CHUL Research Centre, Quebec, Canada). A mouse monoclonal antibody to human progesterone receptors was obtained commercially (Novocastra PGR antibody; Novocastra laboratories, Newcastle, Tyne, UK). In addition, mouse monoclonal antibodies which recognised both 'A' and 'B' isoforms of the progesterone receptor, or solely the 'B' isoform of the receptor, were kindly supplied by Dr C.L. Clarke, University of Sydney, Sydney, Australia. Monoclonal mouse anti-human TIMP-1 antibody was obtained commercially (TIMP-1 (Ab-1); Cambridge Bioscience, Cambridge, Cambs, UK).

3.1.3 Source of Nucleic Acid Probes

A 1.5 kilobase (kb) complementary DNA (cDNA) construct, corresponding to nucleotide 542 to the last nucleotide of the open reading frame (2124), of the human LH receptor (Minegishi *et al.*, 1990) in pBluescript (Stratagene, Cambridge, Cambs, UK), was kindly supplied by Dr M. Atger of the Faculté de Médècine de Bicêtre, Université Paris-Sud, Le Kremlin-Bicêtre, France. A 1.2 kb cDNA construct of human 3β-HSD in pIBI25 was kindly supplied by Prof J.I. Mason, Dept of Clinical Biochemistry, University of Edinburgh, Edinburgh, UK. A 600 bp cDNA fragment of bovine $P450_{scc}$ was obtained from Prof A.S. McNeilly, MRC Reproductive Biology Unit, Edinburgh, UK.

The following nucleic acid probes were also used: a 0.7 kb fragment of human MMP-1 in pBluescript; a 1.6 kb fragment (6-1576 bp) of human MMP-2 in pGEM 4Z; a 1.3 kb fragment (759-2105 bp) of human MMP-9 in pGEM 4Z; full-length human TIMP-1 in pGEM 4Z; full-length human TIMP-2 in pGEM 4Z; a 0.2 kb fragment (400-600 bp) of human TIMP-3 in pBluescript. The probes to MMP-2, MMP-9, TIMP-1 and TIMP-2 were kindly provided by British Biotech Pharmaceuticals Ltd., Oxford, Oxon, UK. Probes for TIMP-3, and MMP-1 were purchased from University Technologies International Inc. A cDNA probe to human β-actin was obtained by PCR using commercial primers and template (Clontech, Palo Alto, CA, USA). The oligonucleotide fragment used to detect 18S RNA was obtained from Dr J. Brooks (MRC Reproductive Biology Unit, Edinburgh, UK).

3.2 Immunohistochemistry

3.2.1 Preparation of Slides

Glass slides (BDH Laboratory Supplies, Poole, Dorset, UK) were cleaned in hot soapy water. They were then washed in 0.1 M HCl, rinsed in ribonuclease (RNAse)-free water and washed in ethanol. When RNAse-free slides were prepared these were then placed in metal slide racks and baked for 2 h. The slides were then coated with poly-L-lysine (50 μg/l), wrapped in a sterile paper towel, dried at 55 °C, and stored in RNAse-free sterile boxes until use.

3.2.2 Preparation of Fixed Tissue Sections

Five micron sections of paraffin wax-embedded tissues were cut onto poly-L-lysine-coated slides and dried overnight at 50 °C. Slides were dewaxed in xylene for 10 min and, rehydrated through graded alcohols into distilled water. If preliminary experiments indicated that antigen retrieval techniques were required to optimise specific primary antibody binding, these were performed at this stage.

3.2.3 Preparation of Frozen Sections

In the case of frozen tissue, six micron sections were cut from the frozen block (**2.1.3**) onto poly-L-lysine-coated slides. The sections were dried briefly at 37 °C and stored at -70 °C until use. Prior to immunohistochemistry, they were fixed at 4 °C in 15% (v/v) aqueous picric acid, containing 2% (w/v) paraformaldehyde, pH 7.4, for 10 min and washed in 0.1 M phosphate buffered saline (pH 7.4) for 20 min at 4 °C. From this point onwards the frozen sections followed the same immunohistochemical procedure as the fixed sections.

3.2.4 Antigen Retrieval

Treatment of the tissue sections to expose masked antigens involved either proteolytic digestion or microwave antigen retrieval. Where proteolytic digestion was required, the slides were incubated in 0.1% (w/v) trypsin with 0.1% (w/v) calcium chloride, buffered to pH 7.4 with 0.25 M Tris HCl, for 30 min at 37 °C. The sections were then washed in 0.05 M Tris buffered saline pH 8.0 (TBS) before the next stage of the immunohistochemical procedure. Where microwave antigen retrieval was required, the technique of Shi *et al.* (1993) was used. Sections were microwaved at full power (1000 W) in 0.01 M citrate buffer (pH 6) for 10 min and left to stand for 20 min. The sections were then washed in TBS before the next stage of the immunohistochemical procedure.

3.2.5 Blocking Endogenous Peroxidase

Where a horseradish peroxidase (HRP) detection system was used, endogenous peroxidase activity was blocked at this stage. Endogenous peroxidase activity was blocked with 2% (v/v) H_2O_2 in 60% (v/v) methanol for 30 min at room temperature. The slides were then rinsed in TBS before the next stage of the immunohistochemical procedure.

3.2.6 Immunohistochemical Procedure

Tissue was permeabilised with 0.1% Triton-X100 in TBS for 10 min. After washing in TBS, the area of the slide around the tissue section was dried and the slides were transferred to a humid chamber. Non-specific binding was blocked by incubating the sections with serum diluted 1:5 in TBS containing either 4% or 5% (w/v) bovine serum albumin (BSA) for 20 min at room temperature. Normal goat serum (NGS) (SAPU, Carluke, Lanarkshire, UK) was used if the primary antibody was raised in a rabbit and non-immune rabbit serum (NRS) (Dako Ltd.) was used if the primary antibody had been raised in a mouse. Excess blocking buffer was then removed and the primary antibody diluted in TBS, or diluted in 20% normal serum in TBS, was added to the section. The optimal dilution and conditions of the primary antibody had been determined in previous experiments. The sections were incubated with the primary antibody for 18 h at 4 °C in a humid chamber.

The slides were washed twice in TBS for 5 min and then incubated with the secondary antibody (diluted either 1:100 or 1:500 in TBS) for 30 min at room temperature. Biotinylated goat antibodies to rabbit immunoglobulins (Dako Ltd.) were used if the primary antibody was raised in a rabbit and biotinylated rabbit antibodies to mouse immunoglobulins (Dako Ltd.) were used if a mouse was the source of the primary antibody. The slides were washed again in TBS for 5 min and the avidin-biotin (AB) enzyme complex was added to the section for 30 min at room temperature. The detection enzyme linked to this complex was either HRP (AB-HRP Kit; Dako Ltd.) or alkaline phosphatase (AP) (AB-AP Kit; Dako Ltd.).

3.2.7 Colouration of Sections

Excess AB enzyme complex was washed off in TBS for 5 min twice. The sections were then coloured. Where HRP was used as the detection enzyme, antibody binding was visualised using diamino-benzidine (DAB) as a substrate, which gives a stable brown end product (Vector Laboratories, Peterborough, Cambs, UK). Where AP was used, the substrate was either a kit to give a red end product (Alkaline Phosphatase Substrate Kit I; Vector Laboratories), or nitroblue tetrazolium chloride (NBT) which gives a blue end product. When the colouration reaction was complete, the slides were washed in water and counterstained with haematoxylin. If NBT was used as the substrate, the sections were not

counterstained with the purple haematoxylin dye. The slides were then dehydrated through increasing concentrations of alcohol, cleared in xylene and mounted with non-aqueous mounting medium (Pertex; Cellpath, Hemel Hempstead, Herts, UK).

3.2.8 Staining of Tissue Sections

Sections were counterstained with haematoxylin for 20 s to 3 min, and washed with tap water. They were then looked at by light microscopy. If the staining was inadequate, they were stained with haematoxylin for a further minute and rechecked. If the staining was too intense the slides were cleared for 20 s in 1% acid alcohol and restained with haematoxylin. When the haematoxylin staining was adequate, the slides were dehydrated through graded alcohols, cleared in xylene and mounting with Pertex mounting medium.

Sections for morphological study were stained for haematoxylin and eosin. In this case the slides were cleared in xylene for 10 min then dehydrated through graded alcohols, 100% ethanol (2 min), 95% ethanol (2 min), 70% ethanol (2 min), into water. The slides were stained with haematoxylin as above, washed in tap water and stained with eosin for 10 s. After thorough washing, the slides were dehydrated through graded alcohols, cleared in xylene and mounted.

3.2.9 Negative Controls

Where the primary antibody was a polyclonal rabbit antibody, polyclonal rabbit immunoglobulin G (IgG) (Dako Ltd.) at the same antibody concentration, or rabbit serum with an equivalent immunoglobulin concentration (Dako Ltd.), was used in place of the primary antibody, in serial sections, as a negative control. Where the primary antibody was a monoclonal mouse antibody, the primary antibody was replaced by mouse IgG (Vector Laboratories; Dako Ltd.), at the same concentration (typically 5 μg/ml) in serial sections as negative controls. In each case, further serial sections, where the primary antibody was omitted were used as additional negative controls.

3.3 Western Blotting

3.3.1 Preparation of Blot

In order to extract proteins from snap-frozen tissue, the tissue was cut into small pieces and homogenised in 0.1% sodium dodecyl sulphate (SDS) on ice. After sonication, the protein content was estimated by the method of Bradford (1976). Two hundred micrograms of protein was denatured by boiling in sample buffer [4% (w/v) SDS, 40% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.04% (w/v) Bromophenol blue, 10 mM EDTA, 125 mM Tris, pH 7.0] and separated by electrophoresis in fresh running buffer [0.1% (w/v) SDS, 50 mM Tris HCl, 0.4 M glycine] on an 11% polyacrylamide gel at 180 V, for 4 h at 10 °C. Proteins were electrophoretically transferred onto nitro-cellulose membrane (Amersham International, Aylesbury, Bucks, UK) in transfer buffer [25 mM Tris HCl, 0.2 M glycine, 20% (v/v) methanol] at 50 V, overnight at 4 °C. The following morning, the voltage was increased to 100 V for 90 min. After transfer, the blot was washed briefly in TBS containing 0.05% (v/v) Tween (TBST) and incubated in 10% (w/v) dried skimmed milk powder (Marvel; Premier Beverages, Stafford, Staffordshire, UK) in TBST.

3.3.2 Visualisation of Proteins

The blots were incubated with the primary antibody, at a concentration of 2 μg/ml, in TBST for 1 h at room temperature. After washing in TBST, the membranes were incubated with biotinylated rabbit anti-mouse immunoglobulins, at a dilution of 1:3000 in TBST, for 1 h at room temperature. After further washing in TBST, the membranes were incubated with streptavidin AP solution (Dako Ltd.) for 1 h. Antibody binding was visualised, after washing in TBST, using a red chromogen (Alkaline Phosphatase Substrate Kit I; Vector Laboratories). After the bands were visible, the reaction was stopped in water for 10 min. The blots were dried on filter paper and photographed. Molecular weight markers (Biorad Laboratories, Hemel Hempstead, Bucks, UK) were run in an adjacent lane to calculate the weight of the detected proteins.

3.4 *In situ* Ligand Binding

3.4.1 Ligand Binding Reaction

In situ ligand binding was performed using a modification of the method described by Molenaar *et al.* (1993). Frozen sections of 5 μm were cut onto poly-L-lysine-coated slides (**3.2.1**) and stored at -70 °C until use. They were quickly thawed and incubated in binding buffer (50 mM HEPES, 5 mM $MgCl_2$, 0.3% (w/v) BSA, pH 7.4) at room temperature for 20 min. The area of the slide around the tissue section was dried, to remove excess buffer, and the slides were transferred to a humid chamber. One hundred microlitres of binding buffer, containing 10 000 c.p.m. of iodinated LH (Chelsea Reagent) (**3.1.1**), was added for 2 h at room temperature. As negative controls serial sections were incubated with binding buffer containing 10 000 c.p.m. iodinated LH with excess (20 IU) cold hCG (Profasi).

3.4.2 Visualisation of Ligand Binding

The slides were briefly washed four times in 0.05 M Tris pH 7.4 at 4 °C, dipped in distilled water, and allowed to dry for 3 h at 4 °C. The slides were warmed to 37 °C on a slide warmer and dipped in photographic emulsion (Kodak NTB-2; IBI Ltd, Cambridge, Cambs, UK), heated to 37 °C, using a glass dipping chamber. They were then allowed to dry in the vertical position in a darkened moist box for 2 h at room temperature. The sections were then transferred to an air-tight slide box containing silica desiccant and stored at 4 °C for 3 days in the dark. The slides were developed at 15 °C in the dark in freshly filtered developer (Kodak D-19; IBI Ltd) for 4 min, washed and fixed for 15 min (Kodak Unifix; IBI Ltd) (**3.7.6**). The slides were then washed for 20 min in running tap water, counterstained in haematoxylin, dehydrated through graded alcohols and mounted in Pertex mounting medium (**3.2.8**).

3.5 Nucleic Acid Probe Preparation

3.5.1 Transfection and Amplification

The plasmids were transfected into competent E. coli (HB101; Clontech) using the heat shock technique (at 42 °C), according to the manufacturer's instructions.

The bacteria were then added to SOC medium [2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose] (Bio101 Inc., La Jolla, CA, USA) and incubated on a shaking platform at 37°C for 1 h. Fifty microlitres of these cultures were spread on sterile plates containing LB agar (Bio101 Inc.) and ampicillin (50 μg/ml) and incubated overnight at 37 °C. The following day individual colonies were identified and subcloned on ampicillin-agar plates. One clonal colony was added to LB broth (Bio101 Inc.) containing 50 μg/ml ampicillin. This culture was incubated overnight on a shaking platform at 37 °C. The bacterial solution was only used if the control tube, containing only LB broth and ampicillin, was clear when examined the following morning. Plasmids were extracted from these bacterial cultures.

In some situations, PCR fragments were inserted into plasmid vectors. Here, fresh PCR products were ligated into a cloning vector (PCR II Vector; Invitrogen, San Diego, CA, USA), using a DNA ligase reaction at 14 °C, using a commercial cloning kit (TA Cloning Kit; Invitrogen). The plasmid was transfected into competent cells (as supplied) by heating to 42 °C for 2 min, in the presence of β-mercaptoethanol, and immediately placing on ice. Cells, containing the plasmid, were selected on ampicillin (50 μg/ml)-containing X-galactose-coated agar plates. Disruption of the *lacZ*a gene fragment in the vector gives white colonies and implies successful ligation of the PCR fragment. White colonies were subcloned, and one subcloned colony was added to lysis buffer [20 mM Tris-HCl, pH 8.4, 2 mM EDTA, pH 8.0, 1% (v/v) Triton-X 100] and heated to 95 °C for 5 min. This released the DNA to allow the detection of the plasmid insert in that clone by direct lysis PCR (**3.5.4**).

3.5.2 Purification and Stock Preparation

Plasmids were extracted using the Wizard 373 DNA Purification System (Promega). Briefly, the bacterial cultures obtained above were centrifuged at 3000 revolutions per minute (r.p.m.) for 20 min at room temperature and the supernatant discarded. Cells were resuspended in buffer [50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNAse A], lysed with 0.2 M NaOH and 1% SDS and neutralised with 1.32 M potassium acetate, pH 4.8, prior to centrifugation at 13 000 r.p.m at 4°C. The supernatant was mixed with DNA purification resin (as supplied) and the DNA was trapped in the minicolumn supplied with the kit. The DNA was washed with buffer [200 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl,

pH 7.5] and eluted from the minicolumn by adding water at 72°C for 1 min, then pulse centrifuged at room temperature for 1 min. The elutant was reapplied to the column, and collected by pulse centrifugation again, prior to visualisation.

The plasmids were visualised by ethidium bromide staining on an 0.8% agarose gel run at 100 V. Purity and concentration were assessed by comparison with a commercial plasmid DNA standard (pBR322; Pharmacia, Uppsala, Sweden) of known concentration. In some cases, the plasmid DNA was further concentrated by precipitation with 3 M sodium acetate, pH 5.5 and ethanol at -20°C for 2 h. After precipitation, the DNA was collected by centrifugation at 13 000 r.p.m. for 20 min at 4 °C. The DNA pellet was washed with 70% ethanol, air-dried and reconstituted in sterile water.

In each case, prior to plasmid purification, further bacterial stocks were prepared. An aliquot of each culture was added to cold 50% (v/v) glycerol and mixed thoroughly. These stocks were stored at -70 °C. When required these stocks were thawed and an aliquot applied to agar plates, or broth, containing 50 µl/ml ampicillin. This allowed the further propagation of these plasmid-containing bacterial cells.

3.5.3 Restriction Digestion

The plasmids were cut with the required restriction enzymes. Reaction mixtures containing the plasmid DNA, the restriction enzyme (20-40 U) and commercial reaction buffer (One-Phor-All Buffer PLUS; Pharmacia) were incubated at 37 °C for 1 h. A further 20-40 U of the restriction enzyme was added to each mixture, and they were incubated again at 37 °C for 1 h. The samples were then incubated with proteinase K for 30 min at 37 °C. The reaction mixture was incubated with an equivalent volume of phenol:chloroform and the layers separated by pulse centrifugation at 13 000 for 3 min. The aqueous layer was removed to another tube, mixed with an equivalent volume of chloroform and separated by pulse centrifugation. The aqueous layer was mixed with 6 M ammonium acetate and ice cold ethanol, and precipitated overnight at -20 °C.

The following day, the DNA was collected by centrifugation at 13 000 r.p.m. for 15 min at 4 °C. The pellets were washed by pulse centrifugation in 70 % ethanol and air-dried for 2 h at room temperature. After reconstitution, an aliquot of the cut plasmid DNA was visualised by ethidium bromide staining, after

electrophoretic separation on a 1.2% agarose gel. Uncut plasmid was run on a neighbouring lane to confirm the cutting reaction had been successful.

3.5.4 Polymerase Chain Reaction

PCR was used for the production of cDNA fragments from plasmids, and the identification of fragments in plasmids by direct lysis PCR. Here, the oligonucleotides used were based on the SP6 and T7 sequences sandwiching each insert. A reaction mix containing the reaction buffer [100 mM Tris HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3], the plasmid DNA, 10 mM of each deoxynucleotides, the SP6 primer (5'-AGCTATTTAGGTGACACTATAGA-3', at a concentration of 708 ng/µl), the T7 primer (5'-GTAATACGACTCACTAT-AGGGC-3', at a concentration of 670 ng/µl) (Cruachem, Glasgow, Strathclyde, UK) and Taq polymerase (Perkin Elmer, Branchburg, NJ, USA), made up to 50 µl with sterile water.

In a thermal reactor (Hybaid Ltd., Teddington, Middlesex, UK), the DNA was denatured at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 45°C for 1 min and 72°C for 1 min, and finally 72°C for 10 min. The PCR products obtained were run on a 1.2% agarose gel with ethidium bromide staining and photographed under ultraviolet (u.v.) illumination. A pGEM marker (Promega) was run in an adjacent lane to allow calculation of the size of the DNA fragments detected.

3.5.5 Labelling of cDNA Probes

The cDNA probes for Northern blotting were denatured at 95 °C for 5 min and placed on ice for 1 min. They were then radiolabelled with ^{32}P-dCTP (Amersham International) using a commercial random priming kit (Multiprime DNA Labelling System; Amersham International) for 1 h at 37 °C. The labelling reaction was stopped by adding 5 M NaOH, and the sample was buffered and neutralised 3 min later by the addition of 1 M Tris, pH 8.0 and 1 M HCl according to the manufacturers instructions. This mixture was added to the hybridisation buffer during Northern blotting (**3.6.2**) experiments.

3.6 Northern Blotting

3.6.1 Preparation of Blot

Total RNA was isolated by the method of Chomczynski and Sacchi (1987) using a commercial kit (RNAzol B; Biogenesis, Bournemouth, UK or Tri Reagent; Sigma Chemical), and its concentration was determined by absorption at 260 nm. Total RNA (20-30 μg depending on the experiment) was quickly thawed, added to sample buffer [20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, 70% (v/v) deionised formamide, 6% (v/v) formaldehyde] and heated to 65 °C for 5 min. After incubating on ice, dye solution [7.5% (v/v) Ficoll 400, 0.1% (w/v) Bromophenol blue] and 1μl of ethidium bromide solution (1 mg/ml), were added to the samples. The samples were electrophoresed in a 1.5% formaldehyde-agarose gel in running buffer [20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, pH 7.0] at 32 V for 16 h.

After photographing the gel under u.v. illumination, the separated RNA was transferred to a nylon membrane (Amersham International), by capillary action in 20x SSC (1x SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7), overnight at room temperature. The following day the filter was air-dried and wrapped in cling film. The RNA was then fixed onto membranes by u.v. cross-linkage (Spectronics Corporation, New York, NY, USA). Membranes were stored at 4 °C until use. The molecular size of the transcripts was determined by running commercial RNA markers (Promega) in an adjacent lane.

3.6.2 Hybridisation

Membranes were prehybridised for 2-5 h in 15 ml hybridisation buffer [0.5 M sodium phosphate, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS, 6.7% (v/v) deionised formamide], in rotating glass tubes (Hybaid Ltd.) at 65 °C. The cDNA probe (**3.1.3**, **3.5.4**) was labelled with 50 μCi ^{32}P dCTP (Amersham International) by the random priming method using a commercial kit (**3.5.5**) (Amersham International) and added to the hybridisation buffer. Hybridisation was performed for 20 h at 65 °C in the rotating glass tube in a Hybaid oven (Hybaid Ltd.).

3.6.3 Detection of Hybridisation Signal

The membranes were washed twice at 65 °C with 2x SSC for 15 min and once more with 2x SSC/0.1% SDS at 65 °C for 15 min in a rotating glass tube in the Hybaid oven. The blots were then wrapped in cling film and laid down to a phosphor screen for 24-72 h and visualised using a phosphorimager computer (Molecular Dynamics, Maidstone, Kent, UK).

3.6.4 Reuse of Northern Blots

To confirm accurate loading of RNA, the blots were stripped in stripping buffer (5 mM Tris HCl, 0.3 mM EDTA, 0.1x Denhardt's reagent) for 2 h at 65 °C. The blots were then re-probed for 18S RNA (**3.1.3**) with a ^{32}P end-labelled oligonucleotide which hybridises to 18S RNA as described previously (Brooks *et al.* 1992). In some experiments, the blots were reprobed for β-actin (**3.1.3**) with a labelled β-actin PCR fragment..

3.7 *In situ* Hybridisation

Isotopic *in situ* hybridisation was performed on RNAse-free (**3.2.1**) fixed or frozen sections (**2.2.3**) using ^{35}S-labelled riboprobes.

3.7.1 Preparation of Plasmid

Plasmids containing the cDNA sequence linked to RNA polymerase initiation sites were amplified (**3.5.1**) and purified (**3.5.2**) as described previously. Antisense riboprobes were used to detect specific mRNAs in tissue sections. Sense riboprobes were used as negative controls to detect non-specific binding. Plasmids were linearised by restriction digestion as described previously (**3.5.3**). Antisense and sense riboprobes were generated using T3, T7 or SP6 RNA polymerases depending on the plasmid map and cDNA orientation. The restriction enzyme/RNA polymerase combination used in each instance is reported in each experimental chapter.

3.7.2 Synthesis of Riboprobe

Riboprobes, incorporating ^{35}S-labelled UTP (Amersham International), were synthesised using a commercial kit (Promega). The cut plasmids were used as a template in the following labelling reaction: 5 µl of 5x Transcription buffer (Tsc buffer; Promega), 2 µl of DNA template, 1 µl of 10 mM rATP, 1 µl of 10 mM rCTP, 1 µl of 10 mM rGTP, 1 µl of 0.2 mM rUTP, 1 µl of 100 mM DTT, 5 µl of ^{35}S-UTP (50 µCi), 1 µl of RNAse inhibitor (28 U/µl), 1 µl of RNA polymerase (SP6, T3 or T7), made up to 25 µl with sterile water. This mixture was incubated at 37 °C for 45 min, a further 1 µl of RNA polymerase was added and the mixture was incubated at 37 °C for a further 45 min.

Following this incubation, 2 µl of transfer RNA (tRNA) (11 mg/ml) and 1 µl of RNAse-free deoxyribonuclease (DNAse) (1000 U/ml) were added, and the mixture was incubated at 37 °C for 10 min. After adding sterile water to a final volume of 100µl, an equal volume of phenol/chloroform/isoamyl alcohol (50:49:1) was added, and the solution was vortexed and centrifuged at 13 000 r.p.m for 15 s, at room temperature. The solvent was discarded and the extraction procedure was repeated with chloroform/isoamyl alcohol (49:1). The riboprobe was precipitated with 10 µl of 3 M sodium acetate, pH 5.2 and 250 µl ethanol overnight at -70 °C. After recovery by centrifugation (**3.5.3**), the probe was reconstituted in sterile water. After reconstitution, 1µl of the labelled probe was counted in 3 ml of scintillation fluid (Eco Scint; National Diagnostics, Atlanta, Georgia, USA) using a scintillation counter.

3.7.3 Preparation of Sections

Fixed sections (5 µm) on poly-L-lysine-coated slides (**3.2.1**) were dewaxed in fresh xylene, and rehydrated through graded alcohols (**3.2.2**). The slides were then incubated with proteinase K (5 µg/ml) in buffer [100 mM Tris, 50 mM EDTA, pH 8] for 30 min at 37 °C. Where frozen sections were used, 5 µm sections on poly-L-lysine-coated slides (**3.2.1**) were quickly thawed, fixed in 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, for 5 min at room temperature. The frozen sections were then washed twice in 0.1 M sodium phosphate for 5 min. Both types of slides were then treated identically.

The slides were rinsed first in water and then in 0.1 M triethanolamine (TEA) pH 8 for 5 min at room temperature. After rinsing, the slides were acetylated in

0.25% (v/v) acetic anhydride (BDH Laboratory Supplies) in TEA for 10 min. The slides were then washed in 2x SSC for 2 min twice. They were then dehydrated through fresh graded alcohol solutions, 50% ethanol for 3 min, 70% ethanol for 3 min, 95% ethanol for 3 min and 100% ethanol for 5 min. After a further 5 min wash in 100% ethanol, the slides were then dried under vacuum in a desiccator for 1 h at room temperature. The sections were then transferred to small moist plastic dishes (BDH Laboratory Supplies) for hybridisation.

3.7.4 Hybridisation

One hundred microlitres of hybridisation buffer [50% deionised formamide, 10% dextran sulphate, 1x Denhardt's solution, 0.5 mg/ml yeast tRNA, 10 mM dithiothreitol (DTT), 0.3 M NaCl, 10 mM Tris, 1 mM EDTA pH 8] containing 1 x 10^6 c.p.m. ^{35}S-radiolabelled antisense probe was added to each section. The ^{35}S-labelled sense riboprobe (1 x 10^6 c.p.m) was added to serial sections as a negative control. The slides were covered with a hydrophobic coverslip (Gel Bond; ICN Biomedical Ltd, High Wycombe, Bucks, UK) and incubated overnight at 55 °C in a moist chamber.

3.7.5 Washing of Sections

The following day the coverslips were washed off in 4x SSC. After four 6 min rinses in 4x SSC, the slides were treated with RNAse A (20 μg/ml) in RNAse buffer [10 mM Tris, 1 mM EDTA, 0.5 M NaCl, pH 8] for 30 min at 37 °C. The sections were de-salted by rinsing in 2x SSC/1 mM DTT for 6 min twice, followed by 1x SSC/1 mM DTT for 12 min and 0.5x SSC/1 mM DTT for 10 min twice at room temperature. The slides were then washed for 30 min in 0.1x SSC/1 mM DTT at 70 °C in a shaking water bath. After rinsing in 0.1x SSC/1 mM DTT at room temperature for 5 min, the sections were dehydrated through graded alcohols (50%, 70% and 95% ethanol), containing 1 mM DTT and 0.08x SSC, for 3 min each. The sections were then washed in pure ethanol for 3 min, three times and allowed to dry.

3.7.6 Detection of Hybridisation Signal

The slides were air-dried and gently heated to 37 °C on a hot-plate in the dark. They were then dipped in photographic emulsion (Kodak NTB-2) at 37 °C in a glass dipping chamber. The slides were allowed to dry vertically for 2 h in a light-

tight moist container. They were then transferred to a slide rack and stored at 4 °C for 18-21 days in light-tight boxes containing silica gel. The slides were then developed. They were incubated with freshly filtered developer (Kodak D-19) for 4 min at 15 °C in the dark. The slides were rinsed in water containing 0.1% (v/v) glacial acetic acid and fixed for 15 min (Kodak Unifix) at 15 °C. The slides were washed in water and rinsed in running tap water for 1 h, counterstained in haematoxylin (**3.2.8**), dehydrated through graded alcohols, cleared in xylene and mounted in Pertex mounting medium. The sections were viewed and photographed under dark-field illumination. The localisation of the grains was determined by reference to the section viewed under bright-field.

Chapter 4

Experimental Section I: Functional Effects

4.1 General Introduction

This first section explores the functional capacity of the corpus luteum at different stages of its lifespan. To recap, the main synthetic function of the corpus luteum is the production of progesterone (Behrman *et al.*, 1993). Progesterone secretion increases during the early-luteal phase and reaches a peak in the mid-luteal phase (Fig. 4.1). In the absence of hCG from the implanting blastocyst, progesterone production declines during the late-luteal phase (Fig. 4.1). At the time of menstruation, luteal progesterone production has virtually ceased (Lenton and Woodward, 1988). In a conception cycle, hCG is secreted by the trophoblastic tissue of the conceptus in a logarithmically increasing manner (Lenton and Woodward, 1988; Tovanabutra *et al.*, 1993). This hCG acts directly on the corpus luteum to maintain, and increase, the progesterone output of the corpus luteum in the short term (Stouffer, 1988). Therefore, after luteal 'rescue' with hCG, progesterone synthesis increases during the late-luteal phase (Fig. 4.1) and menstruation is prevented.

The reason that the progesterone output of the corpus luteum changes over the luteal phase is not clear. The time when progesterone production is at its greatest flux is in the late-luteal phase (Fig. 4.1). At this time, there are fundamental differences in progesterone synthesis in the absence or presence of hCG (Lenton and Woodward, 1988). Progesterone synthesis from the corpus luteum is absolutely dependent on gonadotrophic stimulation (Hutchison and Zeleznik, 1984; Fraser *et al.*, 1986). During the normal luteal phase, LH from the pituitary gland is responsible for progesterone synthesis (Hutchison and Zeleznik, 1984). LH binds to and activates a specific glycoprotein receptor (Segaloff and Ascoli, 1993) on the surface of the steroidogenic cells (Bramley *et al.*, 1987). The LH receptor is a classic G-protein coupled receptor linked to adenylyl cyclase (Segaloff and Ascoli, 1993; Rojas and Ciridon, 1996). Ligand binding stimulates the activation of intracellular second messenger cascades (Segaloff and Ascoli, 1993; López Bernal *et al.*, 1995), and the synthesis of cAMP (Eyster *et al.*, 1985)

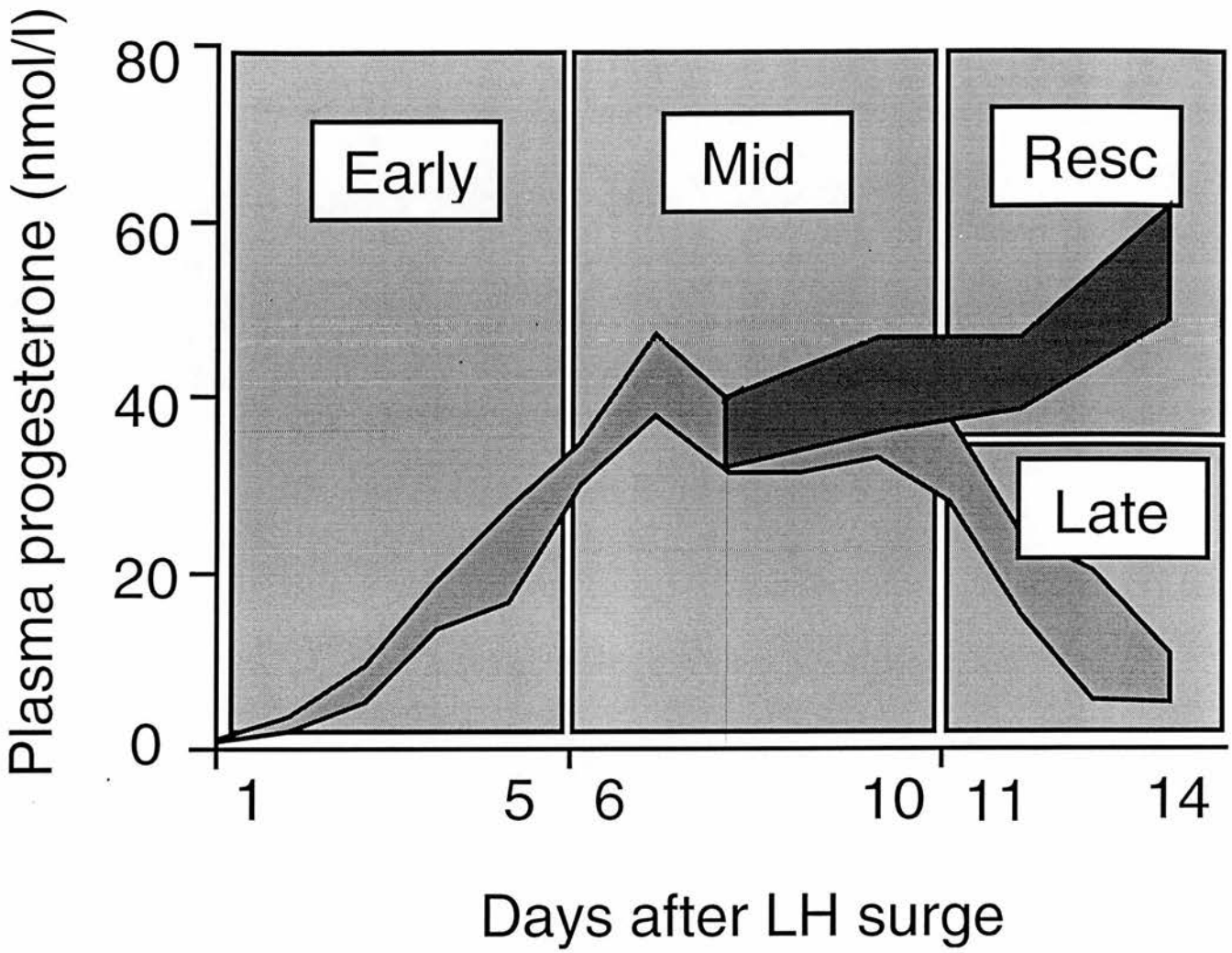


Figure 4.1

Progesterone output of the corpus luteum

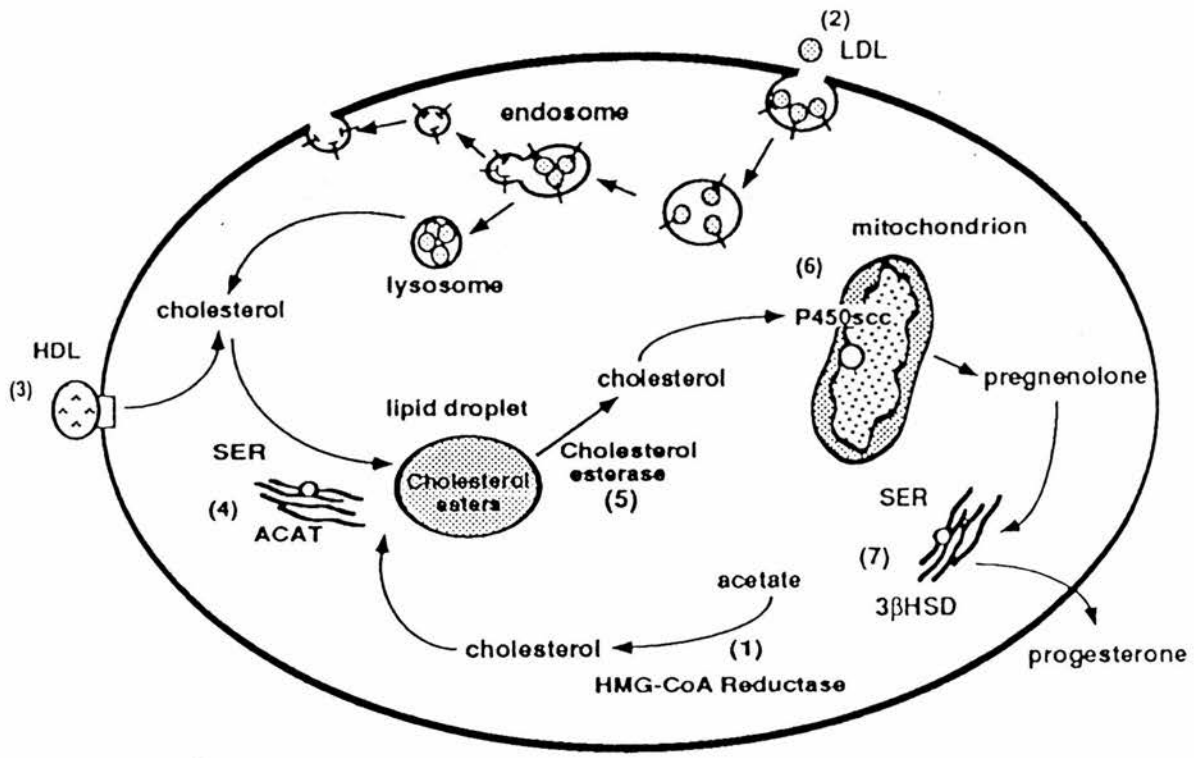
A schematic cartoon indicating progesterone synthesis by the human corpus luteum throughout its functional lifespan. Progesterone output increases in the early-luteal phase (LH+1 to LH+5), is maximal in the mid-luteal phase (LH+6 to LH+10) and declines in the late-luteal phase (LH+11 to LH+14). After luteal 'rescue' with hCG, progesterone production in the late-luteal phase is increased. Progesterone is produced in luteal cells by the actions of the steroidogenic enzymes after LH stimulation.

(Fig. 4.2). Cyclic AMP stimulates the action of the steroidogenic enzymes to convert cholesterol into progesterone (Marsh, 1976) (Fig. 4.2).

The first stage in the conversion of cholesterol to progesterone is the transport of cholesterol from the cell cytosol to the inner mitochondrial membrane, where the first enzyme of the steroidogenic pathway is located (Adashi, 1994; Stocco and Clark, 1996). The transport of cholesterol across the mitochondrial membrane is thought to be under the control of the StAR protein (Clark *et al.*, 1994; Stocco and Clark, 1996). This is likely to be the rate-limiting stage of the steroidogenic pathway (Stocco and Clark, 1996) (Fig. 4.2). The enzyme complex $P450_{scc}$ is located on the inner mitochondrial membrane. It catalyses the conversion of cholesterol into pregnenolone (Simpson and Boyd, 1967). Pregnenolone is converted to progesterone by the action of 3β-HSD (Strauss and Miller, 1991). *De novo* synthesised progesterone is secreted by the steroidogenic cells of the corpus luteum into the local and then systemic circulation (Fig. 4.2). Progesterone acts on its target tissues by binding to a specific nuclear receptor which is responsible for the genomic effects of progesterone (Graham and Clarke, 1997).

For progesterone synthesis to fall during the late-luteal phase, there much be an increasing block to its synthesis somewhere in this pathway. We know that LH withdrawal using GnRH inhibition (Hutchison and Zeleznik, 1984; Fraser *et al.*, 1986) causes progesterone synthesis to cease and induces luteolysis (Fraser *et al.*, 1995b). During the normal luteal phase, the exposure of the corpus luteum to pituitary LH decreases due to a reduced LH pulse frequency (Ellinwood *et al.*, 1984). However this alone is not the cause of functional luteolysis as progesterone synthesis still declines in the presence of artificially maintained LH levels (Hutchison *et al.*, 1986). It is therefore the action of LH which seems to be blocked during the late-luteal phase, when progesterone synthesis is declining.

This decline in progesterone synthesis in the late-luteal phase is prevented by exposure to hCG from the implanting blastocyst (Stouffer, 1988). The molecular mechanisms of how hCG 'rescues' the corpus luteum are not clear. However hCG shares a great homology with LH (Iles and Chard, 1993), albeit with a different glycosylation pattern (Wilson *et al.*, 1990), and exerts its biological actions by activating the LH receptor (Cole *et al.*, 1973). HCG has a longer bioavailability than LH (Wilson *et al.*, 1990) and is present in logarithmically increasing concentrations (Lenton and Woodward, 1988) in early pregnancy. HCG clearly maintains progesterone production by its actions on the LH receptor. However,



Steroidogenic luteal cell, illustrating enzymes and processes involved in synthesis of progesterone. *De novo* cholesterol synthesis (1) proceeds from acetate through action of, primarily, 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG-CoA reductase). Cholesterol and cholesterol esters are also taken up (2) from low-density lipoprotein (LDL) via receptor-mediated endocytosis or (3) from high-density lipoprotein (HDL) through a nonendocytic process. Cholesterol is esterified (4) by acyl coenzyme A:cholesterol acyltransferase (ACAT) for storage in lipid droplets. Cholesterol esterase (CE) effects deesterification (5), and the resultant cholesterol is transported (6) to the inner mitochondrial membrane where cytochrome P450scc cleaves the side chain of cholesterol to produce pregnenolone. Pregnenolone proceeds (7) to the smooth endoplasmic reticulum (SER), where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD).

Figure 4.2

Progesterone synthesis by luteal cells

Cartoon illustrating the synthetic pathways involved in the synthesis of progesterone by luteal cells.

how this happens is not immediately clear. Exposure to its ligand has been shown to down-regulate the LH receptor *in vitro* and *in vivo* (Segaloff and Ascoli, 1993; Peegel *et al.*, 1994) and negate its biological action. The effects of hCG on the LH receptor and the other elements of the steroidogenic pathway at a molecular level are not clear.

4.2 Scope of the Section

The following four chapters aim to systematically address the steroidogenic pathway in the primate corpus luteum throughout its functional lifespan with particular emphasis on luteal 'rescue' during maternal recognition of pregnancy. Each chapter is deliberately written in the form of a scientific paper. This allows each chapter to be read in isolation, in parallel or in series. This format, however, does not facilitate the development of a coherent thesis in this section. At the end of the thesis, therefore, the conclusions of all the experimental chapters will be drawn together in order to develop a paradigm of the corpus luteum during its functional lifespan.

The first part of the steroidogenic pathway to be investigated was the LH receptor itself. The LH receptor was studied during the normal luteal phase, during luteolysis and during simulated maternal recognition of pregnancy. **Chapter 5** addresses the expression and localisation of the LH receptor in the human corpus luteum throughout the functional luteal phase and after luteal 'rescue' with exogenous hCG to simulate early pregnancy. **Chapter 6** addresses the expression of luteal and follicular LH receptors in the primate ovary in response to withdrawal of its ligand and treatment with $PGF_{2\alpha}$. Both of these treatment modalities inhibit progesterone synthesis and initiate luteolysis.

The second part of the steroidogenic pathway to be investigated was the steroidogenic enzymes, and factors downstream from the LH receptor. **Chapter 7** addresses the expression of StAR, $P450_{scc}$ and 3β-HSD in human corpora lutea throughout the functional luteal phase and after luteal 'rescue' with exogenous hCG. **Chapter 7** also addresses the detailed localisation of the 3β-HSD enzyme and its mRNA. **Chapter 6** also addresses the effect of induced luteolysis on the expression and localisation of 3β-HSD in the primate ovary.

The third element of the steroidogenic pathway to be investigated was progesterone itself. There is increasing evidence that progesterone may directly effect its synthesis. **Chapter 8** addresses the autocrine role of progesterone in the corpus luteum by investigating the amount, localisation and nature of progesterone receptors in the corpus luteum. Luteal progesterone receptors were studied throughout the luteal phase and after luteal 'rescue' with exogenous hCG. Their expression in the corpus luteum was compared and contrasted with that seen in a classic progesterone-target tissue, the endometrium.

Chapter 5

LH receptor in the human corpus luteum: lack of down-regulation during maternal recognition of pregnancy

5.1 Abstract

Luteal progesterone production is dependent on LH from the pituitary gland. Despite continuing LH secretion, the human corpus luteum undergoes functional luteolysis unless it is 'rescued' by hCG, produced by the implanting blastocyst. As LH and hCG act through a common receptor, this study sought to determine the expression of the LH/hCG receptor in the corpus luteum during maternal recognition of pregnancy. Corpora lutea were collected at hysterectomy from women in the normal luteal phase and after luteal 'rescue' with exogenous hCG. In each case the corpus luteum was classified according to the date of the LH surge measured in daily urine samples. The expression of the LH receptor was investigated by northern blotting, *in situ* hybridisation and *in situ* ligand binding. LH receptor mRNA and ligand binding activity were detected in corpora lutea from all stages of the luteal phase. LH receptor expression and binding were maintained during maternal recognition of pregnancy, in the presence of exponentially increasing hCG. These data show that the LH receptor is maintained throughout the functional life-span of the human corpus luteum and is not down-regulated during maternal recognition of pregnancy.

5.2 Introduction

Progesterone production by the primate corpus luteum is dependent on circulating LH from the pituitary gland (Hutchison and Zeleznik, 1984; Fraser *et al.*, 1986). Withdrawal of circulating LH results in both structural and functional luteolysis. However, despite the continued secretion of LH, functional luteolysis occurs after 14 days unless hCG is secreted by the implanting blastocyst (Hutchison *et al.*, 1986). Both LH and hCG exert their luteotrophic actions through a common receptor (Cole *et al.*, 1973). The human LH/hCG receptor cDNA has now been cloned and sequenced (Minegishi *et al.*, 1990). It is part of a family of G-protein-coupled receptors, with seven transmembrane regions and a large glycosylated extracellular domain (Segaloff and Ascoli, 1993).

In the presence of increasing hCG, during maternal recognition of pregnancy in women, luteal progesterone production increases, and circulating progesterone concentrations rise (Lenton and Woodward, 1988; Tovanabutra *et al.*, 1993). However, in other species the LH receptor has been shown to undergo desensitisation and down-regulation after exposure to its ligand (Niswender *et al.*, 1985; Segaloff and Ascoli 1993; Peegel *et al.*, 1994). In addition, previous studies investigating the LH receptor in the human corpus luteum have reported low levels of receptor in the corpus luteum of ectopic pregnancy (Rao *et al.*, 1977b; Bramley *et al.*, 1987; Yamoto *et al.*, 1988). It is therefore not clear how the human corpus luteum is able to augment progesterone production during maternal recognition of pregnancy.

This study aimed to investigate the luteal LH/hCG receptor during the process of maternal recognition of pregnancy at the time of menstrual delay. We studied LH/hCG receptor mRNA expression and ligand binding in carefully dated human corpora lutea from throughout the normal luteal phase and after luteal 'rescue' with logarithmically increasing doses of exogenous hCG.

5.3 Specific Materials and Methods

5.3.1 Tissues Studied

Corpora lutea were enucleated at the time of hysterectomy in women undergoing surgery for benign conditions (**2.2**). Four corpora lutea were classified as early-

luteal, four as mid-luteal, four as late-luteal and four were classified as luteal 'rescue' (**2.2.4**). At operation, a piece of tissue was rapidly snap frozen in liquid nitrogen and stored at -70 °C for subsequent RNA extraction (**2.2.3**). Another piece of the corpus luteum was frozen in embedding medium and stored at -70 °C until frozen sections were cut (**2.2.3**). Frozen sections were stored at -70 °C until required. In each case, an endometrial biopsy was also fixed in 4% paraformaldehyde and processed into paraffin wax for luteal phase-dating by tissue morphometry (**3.2.8**).

5.3.2 Northern Blotting

Total RNA was extracted from each tissue sample as described previously (**3.6.1**). Twenty-five micrograms of RNA was separated by electrophoresis in a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane and fixed by u.v. cross-linkage (**3.6.1**). Membranes were then pre-hybridised for 3 hours at 65 °C (**3.6.2**). A human LH receptor probe (**3.1.3**) was labelled with 50 μCi ^{32}P dCTP by the random priming method (**3.5.5**). The membranes were then hybridised overnight at 65 °C (**3.6.2**). After washing (**3.6.3**), the membranes were laid onto a phosphor screen for 36 hours and visualised using a phosphorimager computer (**3.6.3**). To correct for minor differences in RNA loading the blots were stripped (**3.6.4**) and re-probed for 18S RNA using an oligonucleotide probe (**3.1.3**). The molecular size of the detected transcripts was determined by running RNA markers in an adjacent lane (**3.6.1**).

5.3.3 *In situ* Hybridisation

Isotopic *in situ* hybridisation was performed on frozen sections using ^{35}S-labelled riboprobes. The antisense probe was generated from the plasmid vector (**3.1.3**) linearised by HindIII (**3.7.1**) using T3 RNA polymerase (**3.7.2**). The sense probe was used as a negative control. This was generated from the plasmid vector linearised by EcoR1 (**3.7.1**) using T7 RNA polymerase (**3.7.2**).

Frozen sections (**2.2.3**) on poly-L-lysine-coated, RNAse free, slides (**3.2.1**) were thawed, fixed and prepared as described previously (**3.7.3**). One hundred microlitres of hybridisation buffer (**3.7.4**) containing 1 x 10^6 c.p.m. radiolabelled probe was added to each section. The slides were covered with a hydrophobic coverslip and incubated overnight at 55 °C in a moist chamber. The following day the coverslips were washed off, the slides were rinsed and treated with RNAse A

(**3.7.5**). The slides were then washed under increasingly stringent conditions, dehydrated and allowed to dry (**3.7.5**). The slides were then dipped in photographic emulsion and incubated in the dark for 21 days (**3.7.6**). They were developed and fixed at 15 °C in the dark (**3.7.6**). After rinsing in running tap water, the sections were counter-stained with haematoxylin and mounted (**3.2.8**).

5.3.4 *In situ* Ligand Binding

In situ ligand binding was performed on frozen sections (**3.4**). Five micron sections on poly-L-lysine coated slides (**3.2.1**) were quickly thawed and incubated in binding buffer (**3.4.1**) at room temperature for 20 min. Excess buffer was removed and iodinated LH (**3.1.1**) or iodinated LH with excess cold hCG (**3.1.1**) was added to each slide for 2 hours at room temperature (**3.4.1**). The slides were washed, dried and dipped in photographic emulsion (**3.4.2**). After incubation at 4 °C for 3 days in the dark, the sections were developed (**3.7.6**), washed, counterstained and mounted (**3.2.8**).

5.3.5 Analysis of Results

The distribution and number of silver grains was analysed by dark-field microscopy after image capture, using computer-based image analysis systems. To quantify the results of the *in situ* hybridisation, the area proportion of silver grains over the steroidogenic cells was measured in five random fields for each section using an image analysis program (NIH Image 1.55; NIH, MD, USA). Acellular areas, or areas without the steroidogenic cells, were ignored. Only sections from the same run, performed under carefully controlled conditions, were analysed. The results of the *in situ* ligand binding were analysed in a similar fashion except that the grain distribution in this case allowed measurement of absolute numbers of grains. Grains were counted using the Cue-2 image analysis system (Olympus Optical Co. UK Ltd, London, UK). In each case the grain density was compared at each stage of the luteal phase using analysis of variance (ANOVA) with a 5% level of significance.

5.4 Results

5.4.1 Plasma Progesterone Concentrations

The classification of the corpora lutea by serial urinary LH measurement agreed with the luteal-phase dating of endometrial biopsies using the method of Li *et al.* (1988). The plasma progesterone concentrations were 36.4 ±9.3 nmol/l in the early luteal samples, 40.4 ±9.9 nmol/l in the mid-luteal samples and 18.8 ±12.8 nmol/l in the late luteal samples. After luteal 'rescue' by exogenous hCG the plasma progesterone concentrations had increased to 52.8 ±1.1 nmol/l.

5.4.2 Detection of LH/hCG Receptor mRNA

A major 4.5 kb band and minor 6.8 to 7.2 kb bands were detected by northern blotting in the human corpus luteum (Fig. 5.1). These are consistent with the size of the major LH receptor transcripts previously reported in the corpus luteum of primates (Ravindranath *et al.*, 1992a; Nishimori *et al.*, 1995). LH/hCG receptor mRNA could be detected in corpora lutea from all stages of the luteal phase and after luteal 'rescue' with hCG. The LH/hCG receptor mRNA was localised to the steroidogenic cells of the corpus luteum by *in situ* hybridisation (Fig. 5.2*a*). No specific localisation was seen in any of the control sections incubated with the sense probe (Fig. 5.2*b*). Messenger RNA for the LH/hCG receptor could be detected, by *in situ* hybridisation, at all stages of the functional luteal phase and after luteal 'rescue' with exogenous hCG (Fig. 5.2*c*,*d*). No significant differences in the level of LH/hCG receptor expression, as measured by grain density, were found between different stages of the luteal phase (Fig. 5.3). Expression of LH/hCG receptor mRNA during luteal 'rescue' was similar to that seen in the mid-luteal phase corpus luteum.

5.4.3 Detection of LH Binding Sites

Specific binding sites for LH were detected in the steroidogenic cells of the normal corpus luteum (Fig. 5.4*a*,*b*). These binding sites were detected in all corpora lutea from each stage of the luteal phase. In addition, they could also be found after exposure to logarithmically increasing doses of hCG *in vivo*, to simulate maternal recognition of pregnancy (Fig. 5.4*c*). No specific binding of LH was observed in the negative control sections (Fig. 5.4*d*). When the binding sites were quantified, using grain counting, no significant differences were observed at

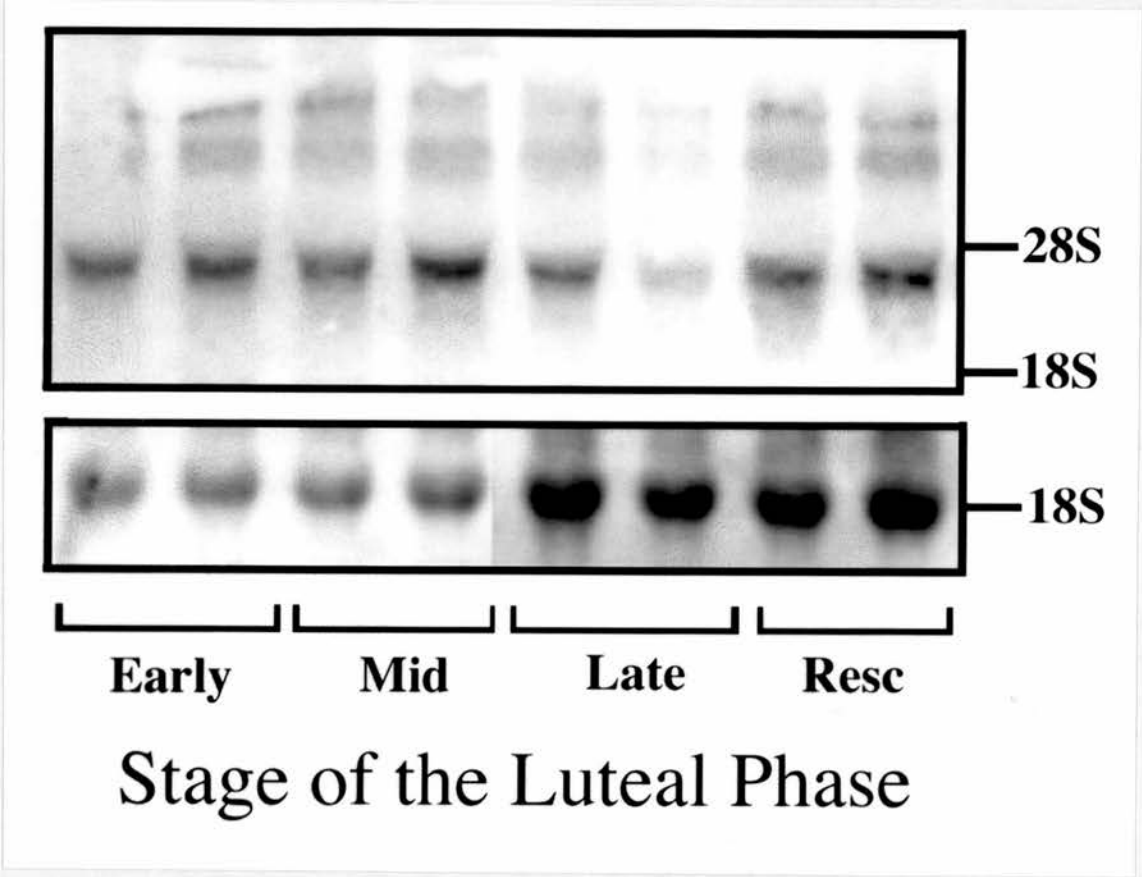


Figure 5.1

Northern blotting for LH receptors in human corpora lutea

Northern blotting for LH receptor in human corpora lutea. The position of the 28S and 18S ribosomal RNA bands are indicated on the right. Expression of 18S RNA is shown to control for differences in RNA loading. LH receptor mRNA could be detected in corpora lutea from all stages of the luteal phase [Early (LH+1 to LH+5), Mid (LH+6 to LH+10), Late (LH+11 to LH+14)] and after luteal 'rescue' with exogenous hCG (Resc).

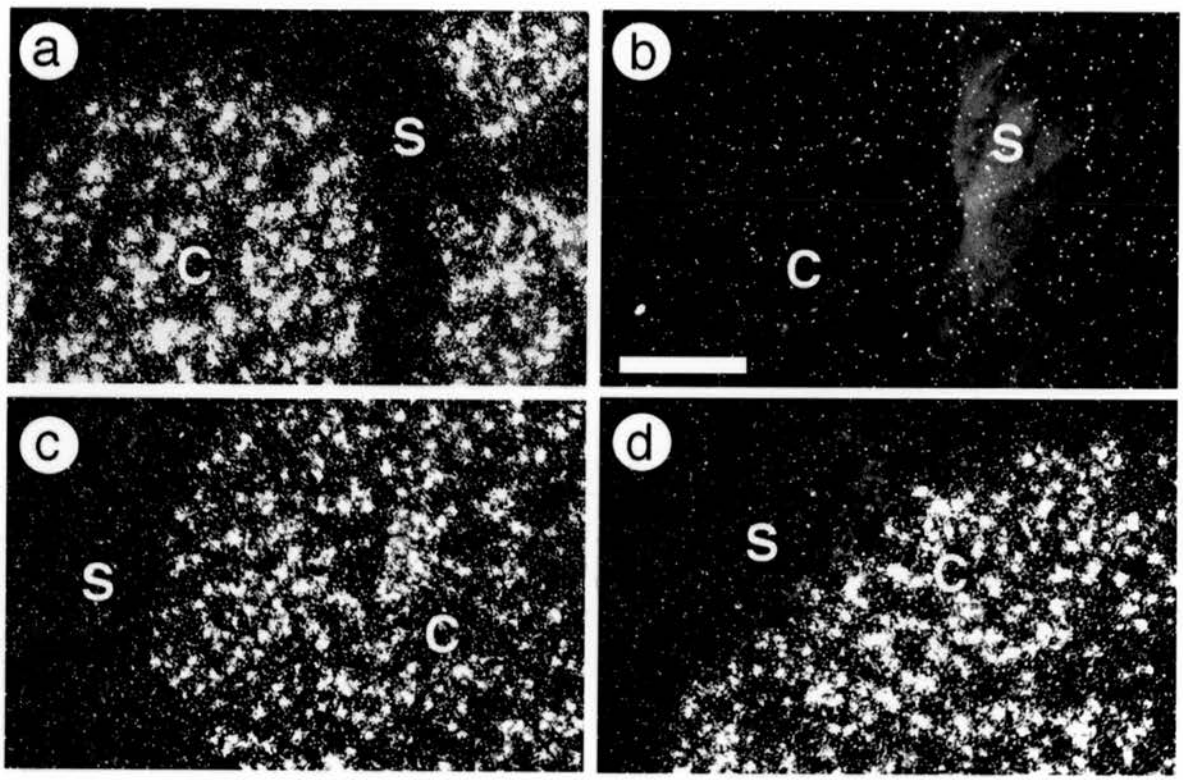


Figure 5.2

In situ hybridisation for LH receptor mRNA in human corpora lutea

In situ hybridisation for LH receptor mRNA in the human corpus luteum: **a)** dark-field of corpus luteum from the early luteal phase, many more grains are seen over the steroidogenic cells (C) than the surrounding stroma (S); **b)** negative control dark-field serial section of (a) after *in situ* hybridisation with the sense riboprobe, showing no difference in background hybridisation between the steroidogenic cells (C) and the surrounding stroma (S); **c)** dark-field late-luteal corpus luteum showing LH receptor mRNA in the steroidogenic cells (C) and not in the stroma (S); **d)** dark-field of LH receptor mRNA in a corpus luteum after 'rescue' with exogenous hCG, expression is maintained in the steroidogenic cells (C) and is absent from the surrounding stroma (S). (Scale bar = 200 μm).

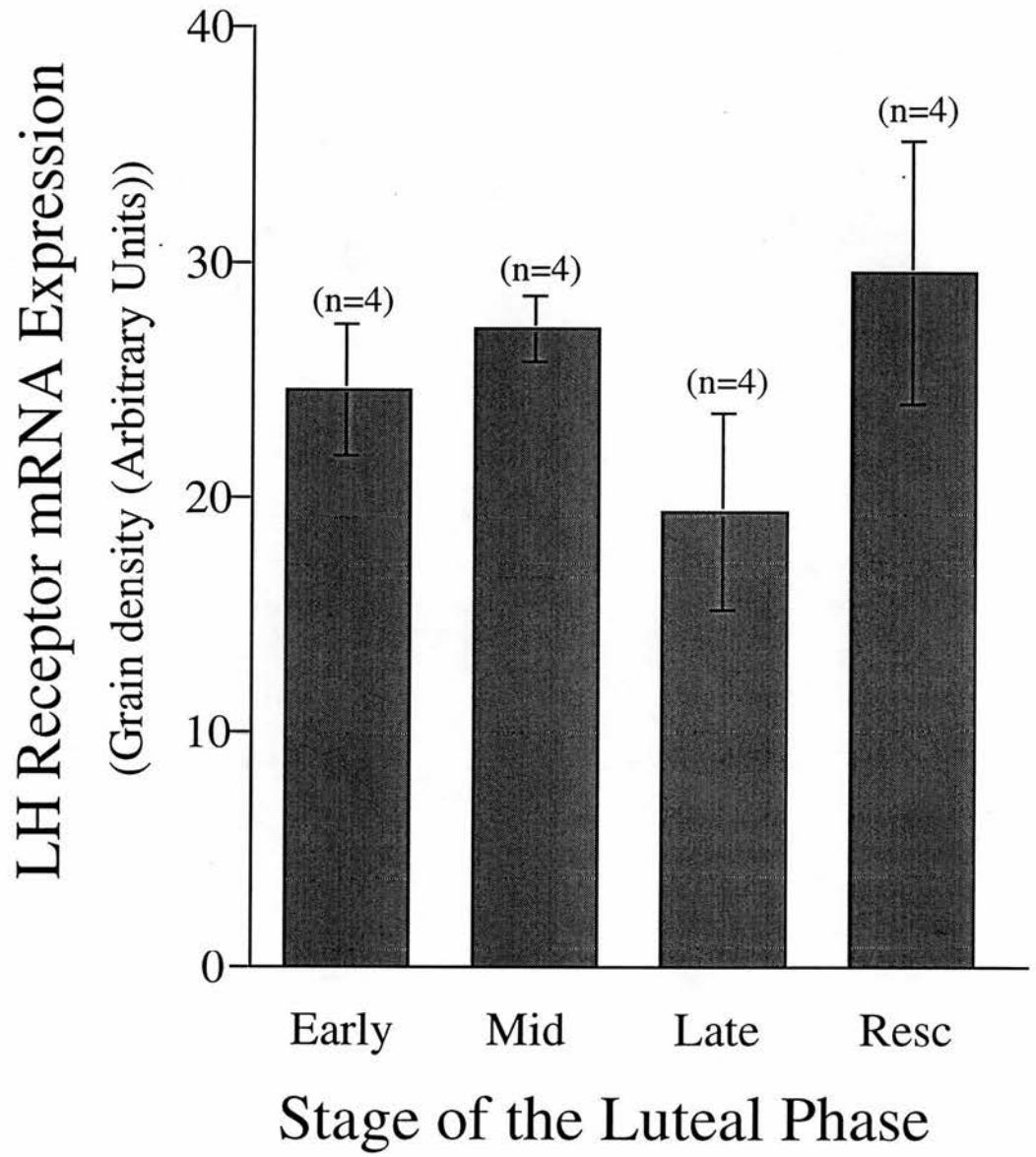


Figure 5.3

LH receptor expression in human corpora lutea

LH receptor mRNA in the human corpus luteum as measured by grain density after *in situ* hybridisation. No differences were seen in the expression of LH receptor message in the early-, mid- and late-luteal phases or after luteal 'rescue' with exogenous hCG. Values are means ± S.E.M.

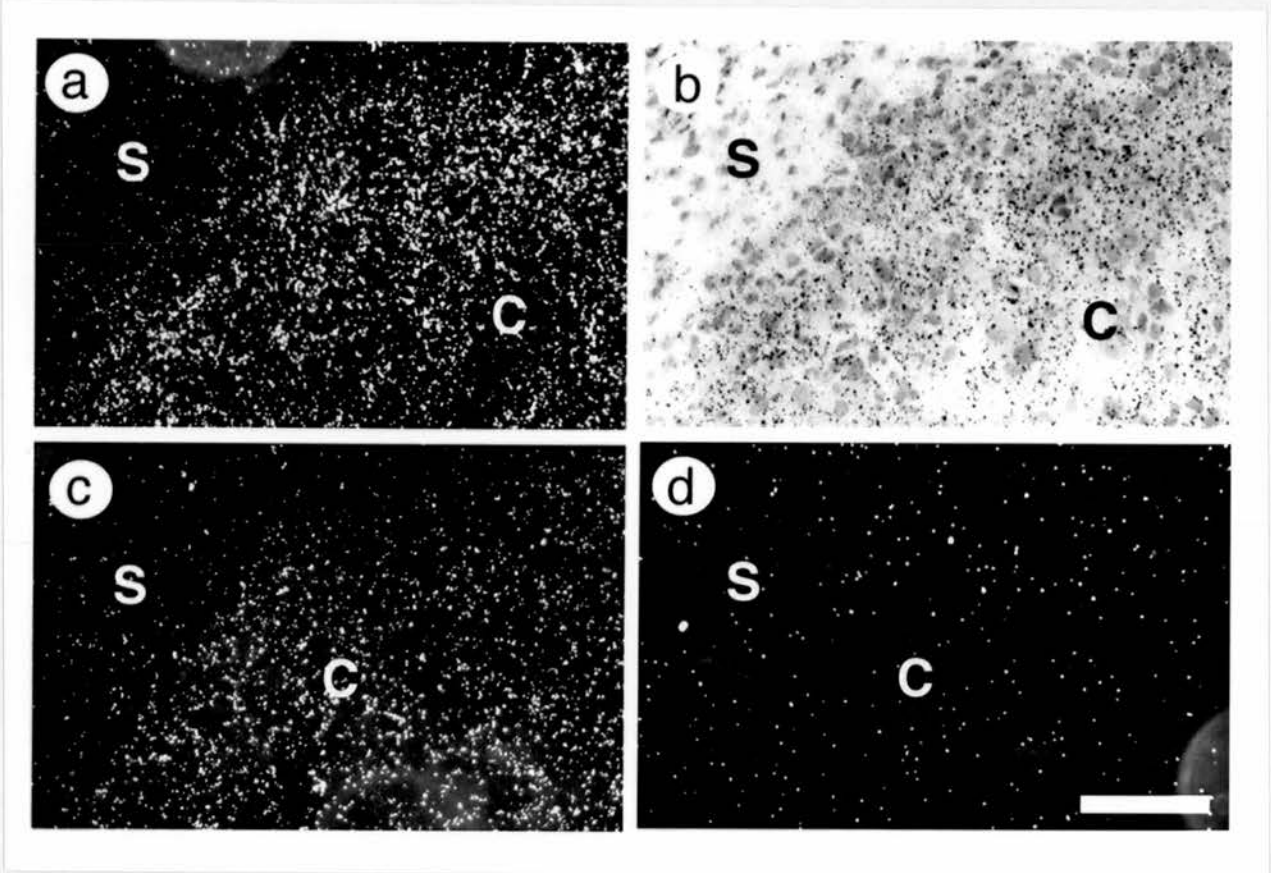


Figure 5.4

***In situ* LH binding in human corpora lutea**

Demonstration of the LH receptor in the human corpus luteum by *in situ* ligand binding: **a)** dark-field of corpus luteum from the mid-luteal phase, many more grains are seen over the steroidogenic cells (C) than the surrounding stroma (S); **b)** light-field of section (a) showing the localisation of the steroidogenic cells (C) and the surrounding stroma (S); **c)** dark-field of LH binding in the corpus luteum after 'rescue' with exogenous hCG, showing continued binding to the steroidogenic cells (C) but not the stroma (S); **d)** dark-field of negative control serial section of (c) showing no specific binding to the steroidogenic cells (C) or the surrounding stroma (S). (Scale bar = 100 μm).

any stage of the luteal phase or after luteal 'rescue' with hCG (Fig. 5.5). The LH/hCG receptor protein, as measured by specific binding, was maintained during maternal recognition of pregnancy at similar levels to the mid-luteal phase.

5.5 Discussion

This paper describes the expression of the LH/hCG receptor in human corpora lutea throughout the functional luteal phase and after luteal 'rescue' with exogenous hCG. Messenger RNA for the LH/hCG receptor has previously been demonstrated in the primate corpus luteum at different stages of the luteal phase (Ravindranath *et al.,* 1992a; Nishimori *et al.,* 1995). Ravindranath *et al.* (1992a) studied the expression of the LH receptor in corpora lutea of cynomolgus monkeys. They reported that LH receptor mRNA increased in the early-luteal phase and was continually expressed, in the corpus luteum, throughout the luteal phase. Our data confirm that the LH/hCG receptor is expressed throughout the functional life-span of the primate corpus luteum.

These observations differ slightly from those of Nishimori *et al.* (1995) who reported a significant reduction in expression of LH receptor mRNA in the late-luteal phase. Although levels of LH receptor mRNA tended to be lower in the late-luteal corpus luteum, this did not reach statistical significance in our study. This is unlikely to be explained by the number of corpora lutea examined as the same number were investigated in each study. We studied the expression of the LH receptor by quantifying the grain density over steroidogenic cells after *in situ* hybridisation. Nishimori *et al.* (1995) used northern blotting of whole gland mRNA to quantify LH receptor expression, and the difference may reflect these different techniques. However, as the LH receptor is not expressed in the corpus luteum after menstruation (Ravindranath *et al.,* 1992a; Nishimori *et al.,* 1995), it is clear that its expression is switched off at the completion of functional luteolysis. As the definition of the late-luteal phase differs in each study, it is possible that the late-luteal glands studied by Nishimori *et al.* (1995) were closer to the completion of functional luteolysis than in our study.

It has been suggested that the stability of transcribed LH receptor mRNA may be decreased to prevent translation into the receptor protein (Lu *et al.,* 1993). We used *in situ* ligand binding to identify the LH receptor protein in the human corpus

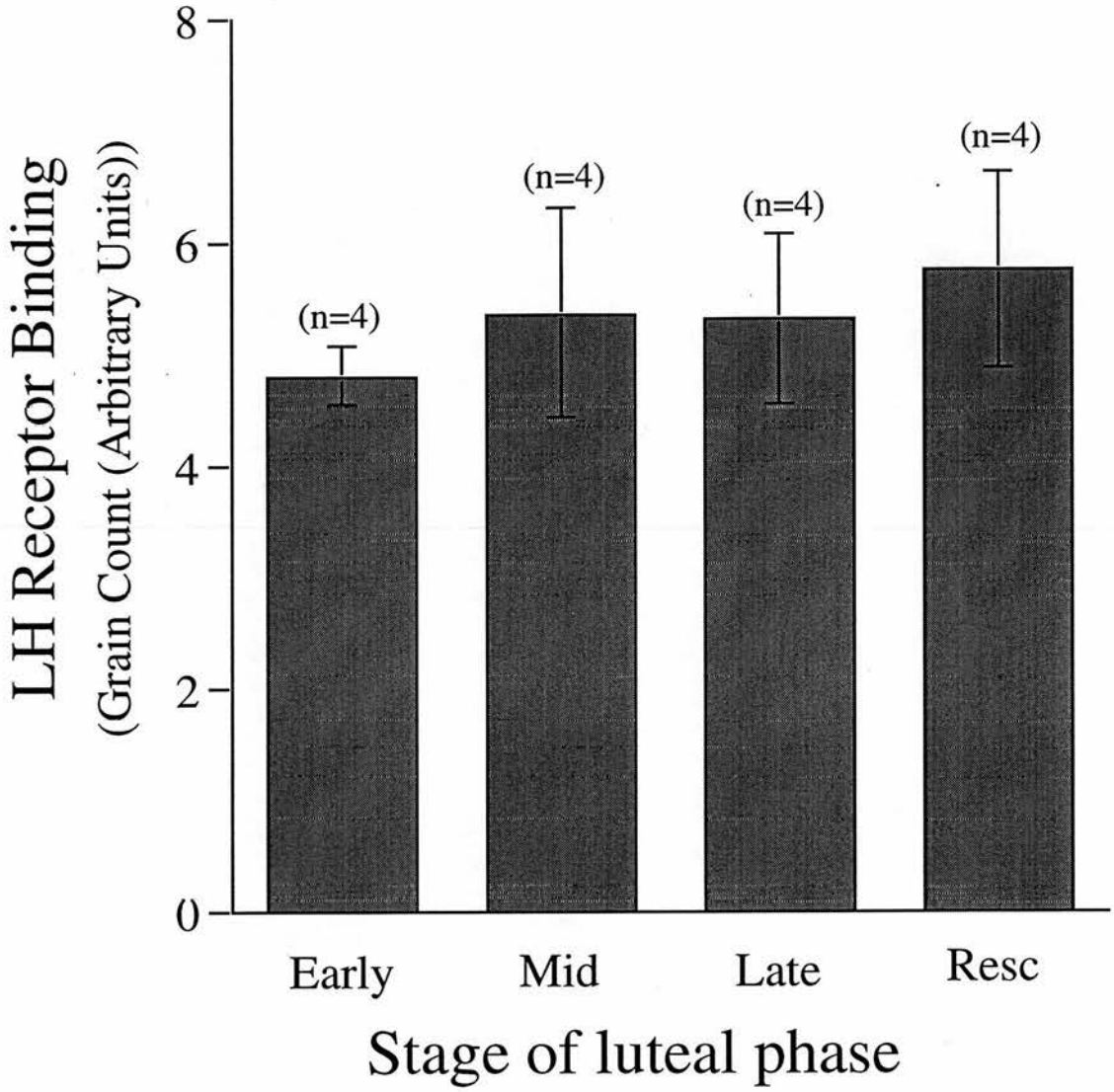


Figure 5.5

LH receptor ligand binding in human corpora lutea

LH receptors in the human corpus luteum as measured by grain counting after *in situ* ligand binding. No differences were seen in the number of LH binding sites in the early-, mid- and late-luteal phases or after luteal 'rescue' with exogenous hCG. Values are means ±S.E.M.

luteum. Numerous studies have demonstrated the presence of LH/hCG receptors in the human corpus luteum using ligand binding assays (Rao *et al.,* 1977b; McNeilly *et al.,* 1980; Shima *et al.,* 1987). Although these studies reported that LH receptor binding was reduced in the late-luteal phase, it has subsequently become clear that when receptor occupancy was taken into account, levels of total receptor are similar throughout the luteal phase (Bramley *et al.,* 1987; Yeko *et al.,* 1989). We have confirmed that the LH receptor protein, in addition to mRNA, is maintained in the corpus luteum throughout its functional life-span.

The cause of functional luteolysis in the primate is not clear (Behrman *et al.,* 1993). The decline in progesterone secretion in the late-luteal phase is not associated with falling serum LH concentrations (Hutchison *et al.,* 1986). This suggests that the corpus luteum is becoming increasingly insensitive to LH. Expression of the LH/hCG receptor is regulated both transcriptionally and post-transcriptionally (Segaloff and Ascoli 1993). However, the continued presence of both the receptor mRNA and protein, as measured by ligand binding, suggest that the luteal LH/hCG receptor is maintained while progesterone production is falling. This is consistent with the findings of Cameron and Stouffer (1982) who compared cell-membrane LH-binding with progesterone production in the macaque corpus luteum. These data suggest that functional luteolysis may be associated with an increasing block to steroidogenesis down-stream from LH/hCG receptor binding.

We found that LH receptor mRNA and protein were maintained during maternal recognition of pregnancy with exogenous hCG. Previous studies have investigated the luteal LH/hCG receptor by binding assay in early human pregnancy (Rao *et al.,* 1977b; McNeilly *et al.,* 1980; Bramley *et al.,* 1987; Dawood and Khan-Dawood, 1994). Concentrations of LH receptors were variable but were much lower than the mid-luteal corpus luteum. In addition, LH/hCG receptor mRNA has now been identified in the corpus luteum of pregnancy (Nishimori *et al.,* 1995). Like receptor binding, levels of mRNA expression were much lower than in the mid-cycle corpus luteum. However, in each case material from the corpus luteum of ectopic pregnancies was investigated. In established pregnancies, maternal recognition of pregnancy has taken place, and although luteal progesterone production is continuing, it is beginning to decline (Tovanabutra *et al.,* 1993). In addition, ectopic pregnancies have sub-optimal serum hCG and progesterone concentrations (Barnea *et al.,* 1986; Ledger *et al.,* 1994) and are

usually associated with vaginal bleeding (Li *et al.,* 1991b). It appears that luteal LH/hCG receptor expression is maintained to a greater degree during maternal recognition of uterine pregnancy than in established ectopic pregnancy.

These data are in agreement with that of Ottobre and Stouffer (1986) who studied LH binding to homogenates of rhesus monkey corpora lutea after exogenous hCG administration. They found that, although the number of available receptors dropped, the total number of receptors remained the same. However, it was not clear if these receptors were membrane bound or if recycling of receptors was occurring. Using *in situ* hybridisation in association with *in situ* ligand binding, we have confirmed the continued presence of the LH/hCG receptor and demonstrated the continued transcription of receptor mRNA. These data give strong evidence that the LH receptor is not down-regulated during maternal recognition of pregnancy in the primate.

In contrast, there is considerable evidence for ligand-induced down-regulation of the LH receptor in other cellular systems. In cell lines expressing the LH receptor, exposure to ligand causes a down-regulation of LH receptor binding (Segaloff and Ascoli, 1993). This loss of ligand binding activity is associated with a loss of the LH receptor mRNA (Hoffman *et al.,* 1991). In the rat corpus luteum, LH receptor mRNA could not be detected 24 hours after ligand-induced down-regulation (Peegel *et al.,* 1994). The LH receptor mRNA expression in the same corpora lutea recovered, but not until 72 hours after a single exposure to ligand. In addition, in the adult rat testis, exposure to hCG resulted in a prolonged down-regulation of the LH receptor message (Pakarinen *et al.,* 1990). In ruminants, administration of hCG was also associated with a marked down-regulation of luteal LH receptors (Niswender *et al.,* 1985). Although the LH receptor can be up-regulated in the growing follicle of the rat (LaPolt *et al.,* 1990), this has not been described in the corpus luteum. In the corpus luteum of non-primate species, it is clear that LH receptors are down-regulated both *in vitro* and *in vivo* by exposure to hCG.

It thus seems likely the effect of hCG on the LH receptor is species specific. Caldwell *et al.* (1980) treated luteal phase rats and women with equivalent doses of hCG. In the rat, both progesterone production and LH receptor content fell significantly, whereas in women, luteal progesterone production increased. The mechanisms of luteolysis and maternal recognition of pregnancy differ in primates and non-primate species (Auletta and Flint, 1988). It appears that, by using an LH-

like chorionic gonadotrophin to maintain progesterone production from the corpus luteum, primates have adapted to overcome down-regulation of the LH/hCG receptor during maternal recognition of pregnancy.

The LH receptor is regulated by other mechanisms in addition to transcription and translation. Desensitisation of the receptor to its ligand, with uncoupling from second messenger systems, has been reported *in vitro* and *in vivo* (Segaloff and Ascoli, 1993). Such desensitisation may explain why increasing doses of hCG are required to maintain progesterone production during pregnancy. In addition, there are multiple transcripts of the LH receptor regulated by alternative splicing (Themmen *et al.,* 1994). Bacich *et al.* (1994), using an ovine model, showed that full length receptor mRNA was a minority of the LH receptor mRNA species detected in the corpus luteum. Most mRNA species coded for truncated or non-functioning receptors. This alternative splicing of the LH receptor has also been reported in other species (Aatsinki *et al.,* 1992; VuHai-LuuThi *et al.,* 1992). However, the functional significance of these transcripts is not yet clear and it is not known whether they are expressed in corpora lutea of primates.

In summary, this study shows that both LH receptor mRNA and protein are maintained in the human corpus luteum during maternal recognition of pregnancy. The lack of down-regulation is further evidence that primates and non-primates exhibit different mechanisms to control function of the corpus luteum.

Chapter 6

Induced luteolysis in the primate: rapid loss of LH receptors

6.1 Abstract

The molecular mechanisms involved in luteolysis are still unclear in the primate. This study aimed to investigate the effect of induced luteolysis on the ovarian LH receptor and the steroidogenic enzyme, 3β-HSD, in the marmoset monkey. Luteolysis was induced in the mid-luteal phase either directly by systemic $PGF_{2\alpha}$, or indirectly by LH withdrawal, using systemic $GnRH_{ant}$ treatment. The LH receptor was studied by isotopic mRNA *in situ* hybridisation and *in situ* ligand binding, and 3β-HSD expression was studied using isotopic mRNA *in situ* hybridisation and immunohistochemistry. Induced luteolysis was associated with a reduction in the expression of LH receptor ($p<0.0001$) and 3β-HSD mRNA, closely followed with a reduction in the LH receptor ($p<0.05$) and 3β-HSD protein levels, within 24 hours. There were no differences in the findings whether luteolysis was induced with $PGF_{2\alpha}$ or $GnRH_{ant}$. This study shows that disparate mechanisms to induce luteolysis in the primate result in an identical rapid loss of the LH receptor and 3β-HSD. In conclusion, induced luteolysis leads to a rapid loss of the steroidogenic pathway in luteal cells.

6.2 Introduction

The molecular mechanisms of luteolysis in the primate, and how the functional and structural integrity of the corpus luteum are lost, are still unclear (Auletta and Flint 1988; Auletta *et al.*, 1990; Behrman *et al.*, 1993). It has recently been shown that cell death by apoptosis (Juengel *et al.*, 1993; Fraser *et al.*, 1995b; Young *et al.*, 1997) and remodelling of the ECM by MMPs (Endo *et al.*, 1993a; Tsang *et al.*, 1995) are likely to contribute to the loss of the structural integrity of the corpus luteum during luteolysis. The molecular mechanisms responsible for the loss of its functional integrity, and its falling progesterone output, however, are still not fully understood.

The primate corpus luteum is dependent on the trophic support of LH from the pituitary gland. LH binds specifically to the LH receptor, a seven transmembrane region, G-protein-coupled receptor (Segaloff and Ascoli, 1993), on the surface of luteal cells, to stimulate steroidogenic enzymes to produce progesterone. Withdrawal of LH results in luteolysis (Hutchison and Zeleznik, 1984; Fraser *et al.*, 1986), whereas hCG, from the implanting blastocyst, acts through the LH receptor to maintain steroidogenesis and 'rescue' the corpus luteum (Stouffer, 1988). In **Chapter 5** we have shown that during luteal 'rescue' the LH receptor is maintained and not down-regulated by its ligand (Duncan *et al.*, 1996a).

There is a significant reduction in luteal LH receptors preceded by reduced receptor mRNA levels after $PGF_{2\alpha}$-induced luteolysis in ruminants (Guy *et al.*, 1995; Smith *et al.*, 1996a). This is thought to contribute to the functional decline of the ruminant corpus luteum. However, in the monkey, LH receptor mRNA increased during the late-luteal phase (Ravindranath *et al.*, 1992a), when progesterone output declined. Similarly, in the human, LH receptor mRNA and binding activity can be detected in the late-luteal corpus luteum (Nishimori *et al.*, 1995; Duncan *et al.*, 1996a), but not in the corpus luteum after menstruation (Ravindranath *et al.*, 1992a; Nishimori *et al.*, 1995). In the primate, functional luteolysis is thought to occur initially in the presence of normal concentrations of LH receptors.

The primate corpus luteum is capable of recovering from a transient withdrawal of gonadotrophic support (Hutchinson and Zeleznik, 1985) but not from natural functional luteolysis (Stouffer *et al.*, 1977). This study investigated the effect of co-ordinated induced luteolysis on LH receptor and 3β-HSD expression in the

primate. The marmoset monkey was used, as luteolysis can be induced by systemic $PGF_{2\alpha}$, although uterine $PGF_{2\alpha}$ is not the natural luteolysin in this species. In addition, luteolysis can be induced by LH withdrawal using $GnRH_{ant}$. We aimed to discover whether $PGF_{2\alpha}$ had the same effect on luteal LH receptors in the primate as the ruminant, and whether induced luteolysis by withdrawal of gonadotrophic support had the same effect.

6.3 Specific Materials and Methods

6.3.1 Tissues Studied

Ovaries from captive-bred common marmoset monkeys (*Callithrix jacchus jacchus*) were studied (**2.3.1**). The stage of the normal ovulatory cycle was determined by serial plasma progesterone estimation (**2.3.1**). Ovaries were collected on day 10 of the luteal phase, from untreated control animals (n=4) and animals treated with $PGF_{2\alpha}$ analogue 24 hours (n=4) previously, or $GnRH_{ant}$ 12 hours (n=2) or 24 hours (n=4) previously (**2.3.2**). Whole ovaries were snap-frozen in embedding medium until frozen sections (5 μm) were prepared (**2.3.3**). In addition, ovaries were also available from control (n=4) and treated animals (n=6) (**2.3.2**) which had been fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin wax (**2.3.3**). Sections (5 μm) were cut onto poly-L-lysine-coated slides (**3.2.1**) for immunohistochemical analysis (**3.2.2**).

6.3.2 *In situ* Hybridisation

Isotopic *in situ* hybridisation was performed on frozen sections using ^{35}S-labelled riboprobes (**3.7**). Antisense and sense LH receptor riboprobes (**3.1.3**) incorporating ^{35}S-labelled UTP were prepared using a commercial kit. The antisense probe was generated from the plasmid vector linearised by HindIII (**3.7.1**) using T3 RNA polymerase (**3.7.2**). The sense probe was used as a negative control. It was generated from the plasmid vector linearised by EcoR1 (**3.7.1**) using T7 RNA polymerase (**3.7.2**). The antisense riboprobe for 3β-HSD mRNA (**3.1.3**) was generated by T7 RNA polymerase (**3.7.2**) after plasmid linearisation by SstI (**3.7.1**).

Frozen sections on poly-L-lysine-coated slides were quickly thawed, fixed, washed, acetylated and dehydrated as described previously (**3.7.3**). The slides

were then dried under vacuum (**3.7.3**) and 100 µl of hybridisation buffer containing 1 x 10^6 c.p.m. radiolabelled probe was added to each section (**3.7.4**). The slides were covered with a hydrophobic coverslip and incubated overnight at 55 °C in a moist chamber (**3.7.4**). The following day the coverslips were washed off, the slides were rinsed and treated with RNAse A (**3.7.5**). The sections were then washed under increasingly stringent conditions with a final wash for 30 minutes in 0.1x SSC at 70 °C (**3.7.5**). The sections were dehydrated (**3.7.5**) and allowed to dry. They were then dipped in photographic emulsion (**3.7.6**) and stored at 4 °C for 18 days in the dark. After developing and fixing (**3.7.6**) the slides were washed in water, counterstained with haematoxylin, dehydrated through graded alcohols and mounted (**3.2.8**).

6.3.3 *In situ* Ligand Binding

In situ ligand binding was performed as described previously (**3.4**). Frozen sections (**2.3.3**) were quickly thawed and incubated in binding buffer (**3.4.1**). Excess buffer was removed and 10 000 c.p.m. of iodinated LH (**3.1.1**) or 10 000 c.p.m. iodinated LH with excess cold hCG (**3.1.1**) was added to each slide (**3.4.1**). The slides were washed and allowed to dry for 3 hours at 4 °C (**3.4.2**). They were then dipped in photographic emulsion (**3.4.2**) and stored at 4 °C for 3 days in the dark. After developing and fixing (**3.7.6**), the slides were washed, counterstained with haematoxylin, dehydrated through graded alcohols and mounted (**3.2.8**).

6.3.4 Immunohistochemistry

Fixed sections were prepared as described previously (**3.2.2**). Non-specific binding was blocked with 20% NGS made up in TBS containing 5% BSA (**3.2.6**). The sections were incubated overnight at 4 °C with the polyclonal rabbit anti-human 3β-HSD antisera (**3.1.1**) diluted 1:1000 in 20% NGS in TBS (**3.2.6**). The following day, the sections were washed and incubated with biotinylated goat anti-rabbit immunoglobulins (**3.2.6**) diluted 1:500 in TBS, for 30 minutes at room temperature. Specific antibody binding was visualised using an AB-AP complex (**3.2.6**) with a chromagen which gave a stable red end product (**3.2.7**). Sections were counterstained with haematoxylin, dehydrated through graded alcohols and mounted (**3.2.8**). Polyclonal rabbit IgG at the same antibody concentration was used in place of the primary antibody, in serial sections, as a negative control (**3.2.9**).

6.3.5 Analysis of Results

The distribution and number of silver grains was analysed by dark-field microscopy after image capture, using computer-based image analysis systems. To quantify the results of the *in situ* hybridisation, the area proportion of silver grains over the steroidogenic cells was measured in five random fields for each section using an image analysis program (NIH Image 1.55). Acellular areas or areas without the steroidogenic cells were ignored. Only sections from the same run, performed under carefully controlled conditions, were analysed. The results of the *in situ* ligand binding were analysed in a similar fashion except that the grain distribution in this case allowed measurement of absolute numbers of grains. In each case the grain density was compared in each treatment group using ANOVA with a 5% level of significance using a commercial statistics computer program (StatView 4.0; Abacus Concepts Inc, Berkeley, CA, USA).

6.4 Results

6.4.1 Plasma Progesterone Concentrations

Progesterone concentrations in the control animals were 330 ±69 nmol/l (mean ±S.E.M.). Functional luteal regression was observed in all animals treated with either the $GnRH_{ant}$ or the $PGF_{2\alpha}$ analogue (Fraser *et al.*, 1995b). $PGF_{2\alpha}$ treatment resulted in a decline in progesterone concentrations to 22 ±6 nmol/l after 24 hours, and treatment with $GnRH_{ant}$ resulted in progesterone concentrations of 13 nmol/l after 12 hours and 23 ±11 nmol/l after 24 hours. All progesterone concentrations after induced luteolysis were within the normal range of follicular phase levels in the marmoset (Smith *et al.*, 1990).

6.4.2 LH Receptor after Induced Luteolysis

Messenger RNA for the LH receptor was detected by *in situ* hybridisation in corpora lutea of marmoset ovaries from the mid-luteal phase (Fig. 6.1*a,d*). No specific signal was present in the negative control sections incubated with the sense riboprobe (Fig. 6.1*c*). LH receptors were localised to individual steroidogenic cells within corpora lutea. No hybridisation signal could be seen in cells without the morphological appearance of steroidogenic cells which expressed 3β-HSD, including endothelial cells (Fig. 6.1*d*). The localisation of LH

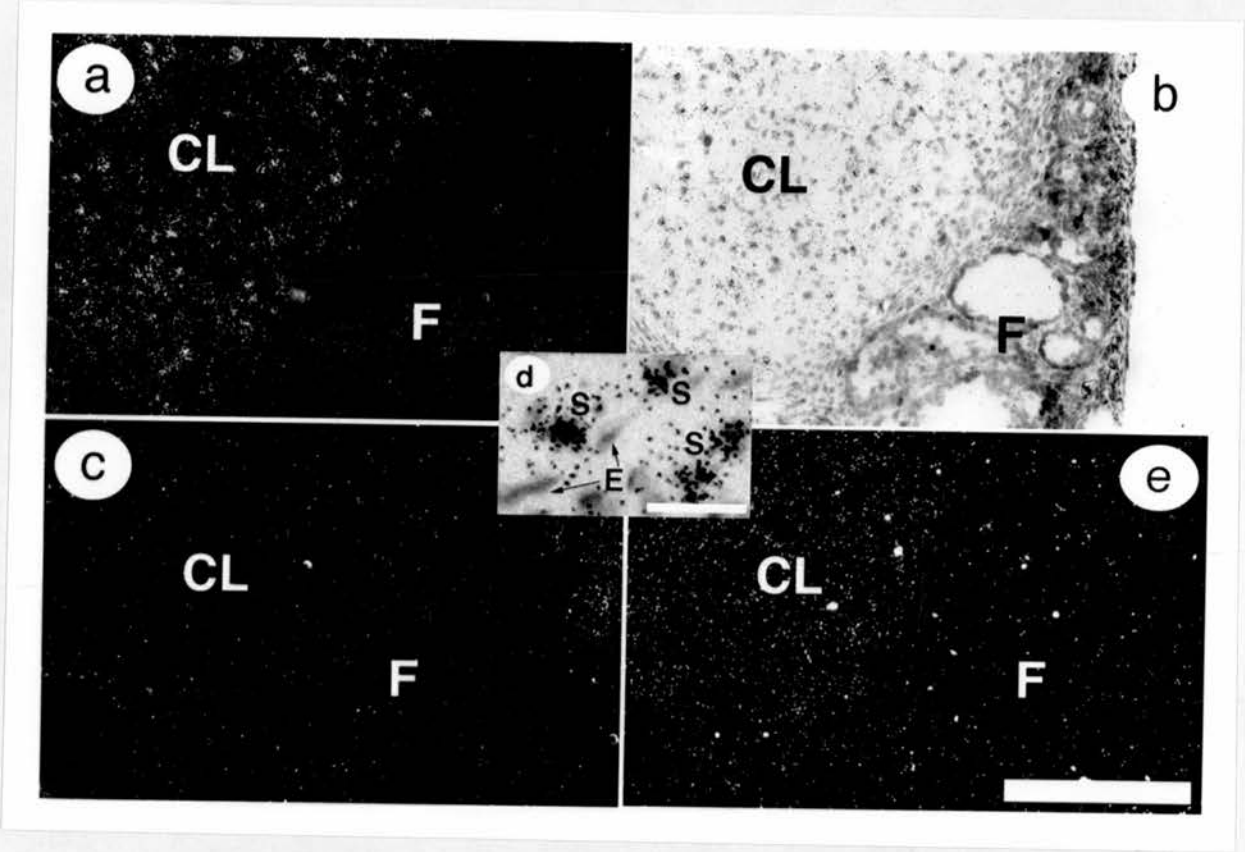


Figure 6.1

LH receptors in the marmoset corpus luteum

LH receptor in the mid-luteal marmoset corpus luteum: **a)** dark-field *in situ* hybridisation of LH receptor mRNA in the control mid-luteal corpus luteum (CL), no signal is seen in the stroma and tiny follicles (F); **b)** light-field serial section of (a) showing the position of the corpus luteum (CL), stroma and tiny follicles (F); **c)** dark-field serial negative control section of (a) using the sense probe for LH receptor mRNA, no signal can be seen in the corpus luteum (CL) or the tiny follicles (F); **d)** light-field higher power (scale bar = 25 μm) section of corpus luteum showing silver grains in steroidogenic cells (S) but not in endothelial-like cells (E); **e)** dark-field serial section of (a) after LH binding studies showing binding over the corpus luteum (CL) and not over the stroma and tiny follicles (F). Scale bar = 200 μm.

receptor binding corresponded to the localisation of LH receptor mRNA (Fig 6.1*e*). No specific binding was seen in negative control sections where excess cold hCG was added.

Messenger RNA for the LH receptor was also expressed in the theca cell layer of antral follicles in ovarian tissue from the mid-luteal phase (Fig. 6.2*a,b*). After induced luteolysis, by $PGF_{2\alpha}$ or $GnRH_{ant}$ administration, LH receptor mRNA disappeared from corpora lutea but was maintained in the theca cell layer of antral follicles (Fig. 6.2*c,d*). Luteal LH receptor mRNA concentrations fell after treatment to very low levels 12 and 24 hours ($p<0.0001$) after induced luteolysis (Fig. 6.3). Luteal LH receptor binding was similar to controls 12 hours after induced luteolysis but was significantly reduced by 24 hours ($p<0.05$) (Fig. 6.3). Although the small numbers at 12 hours precluded statistical analysis, the loss of LH binding appeared to lag behind the loss of LH receptor mRNA (Fig. 6.3; Fig 6.4*a-f*). There were no differences in luteal LH receptor mRNA or binding where luteolysis was induced with $PGF_{2\alpha}$ or $GnRH_{ant}$.

6.4.3 3β-HSD after Induced Luteolysis

Both mRNA (Fig. 6.5*a*) and protein (Fig. 6.5*b*) for 3β-HSD could be detected in corpora lutea of mid-luteal phase ovaries. Twenty-four hours after induced luteolysis, with either $PGF_{2\alpha}$ or $GnRH_{ant}$, no mRNA for 3β-HSD could be detected in corpora lutea (Fig. 6.5*c*), although it could still be detected in the theca cell layer of developing follicles. After 24 hours after induced luteolysis, 3β-HSD protein could still be detected by immunohistochemistry (Fig. 6.5*d*): however, the immunostaining was more patchy and less intense (Fig. 6.5*d*) in each ovary studied.

6.5 Discussion

This study reports the expression of the LH receptor and the steroidogenic enzyme, 3β-HSD, after induced luteolysis in the primate. We have previously reported the expression and localisation of the LH receptor in the human corpus luteum throughout the functional luteal phase and during simulated early pregnancy (Duncan *et al.*, 1996a). In that study, it was not clear that LH receptor mRNA fell during the late-luteal phase. Other studies have suggested either an

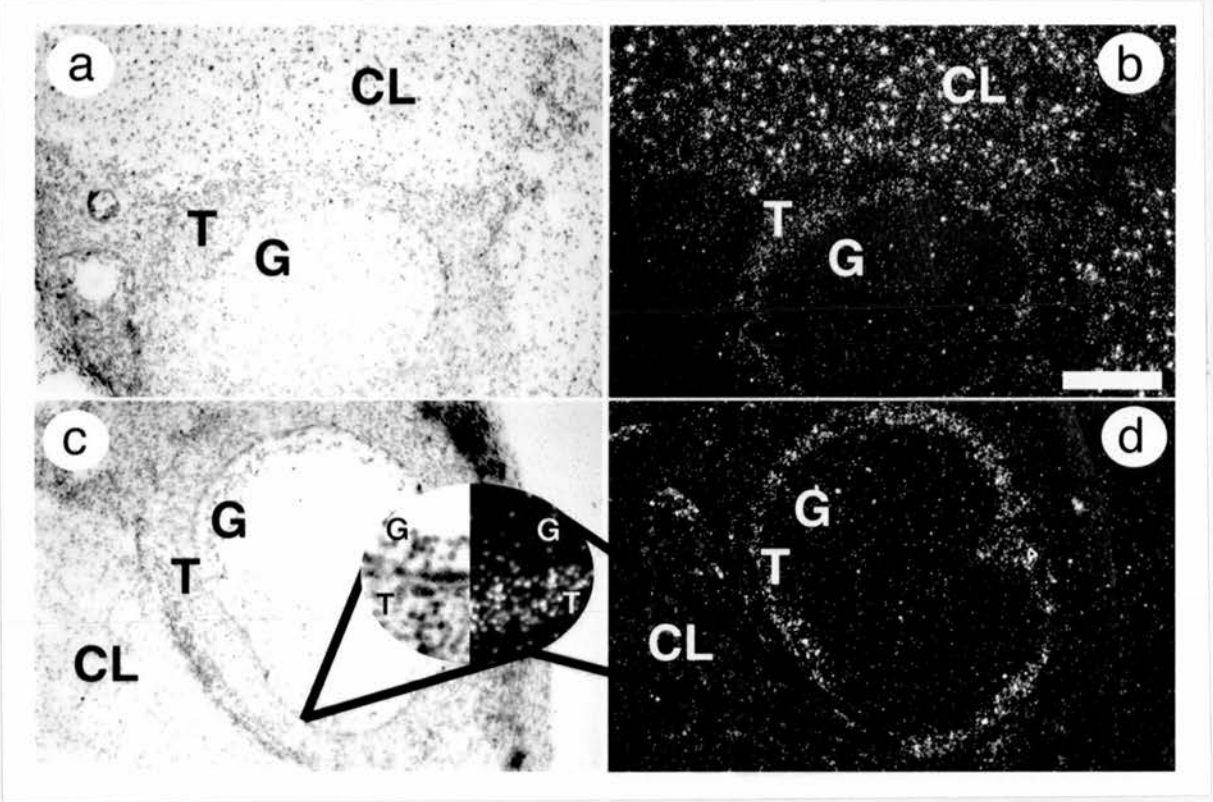


Figure 6.2

Expression of LH receptors in the marmoset ovary after induced luteolysis

LH receptor mRNA in the marmoset ovary after induced luteolysis: **a)** light-field section of a mid-luteal marmoset ovary showing the corpus luteum (CL) and the theca (T) and granulosa cells of an antral follicle; **b)** dark-field section of (a) after *in situ* hybridisation for LH receptor mRNA showing specific signal in the corpus luteum (CL) and theca (T) cells of the follicle, but not the granulosa cells (G) of the follicle; **c)** light-field section of a marmoset ovary 24 hours after induced luteolysis with $GnRH_{ant}$ showing the corpus luteum (CL) and the theca (T) and granulosa (G) cells of an antral follicle; **d)** dark-field section of (c) after *in situ* hybridisation for LH receptor mRNA showing no signal in the corpus luteum (CL) and granulosa cells (G) but maintenance of the signal in the theca cells (T) of the antral follicle. Scale bar = 200 μm. Insert shows higher power view of the follicle wall showing the grains to be localised to the thecal (T) rather than granulosa (G) cells.

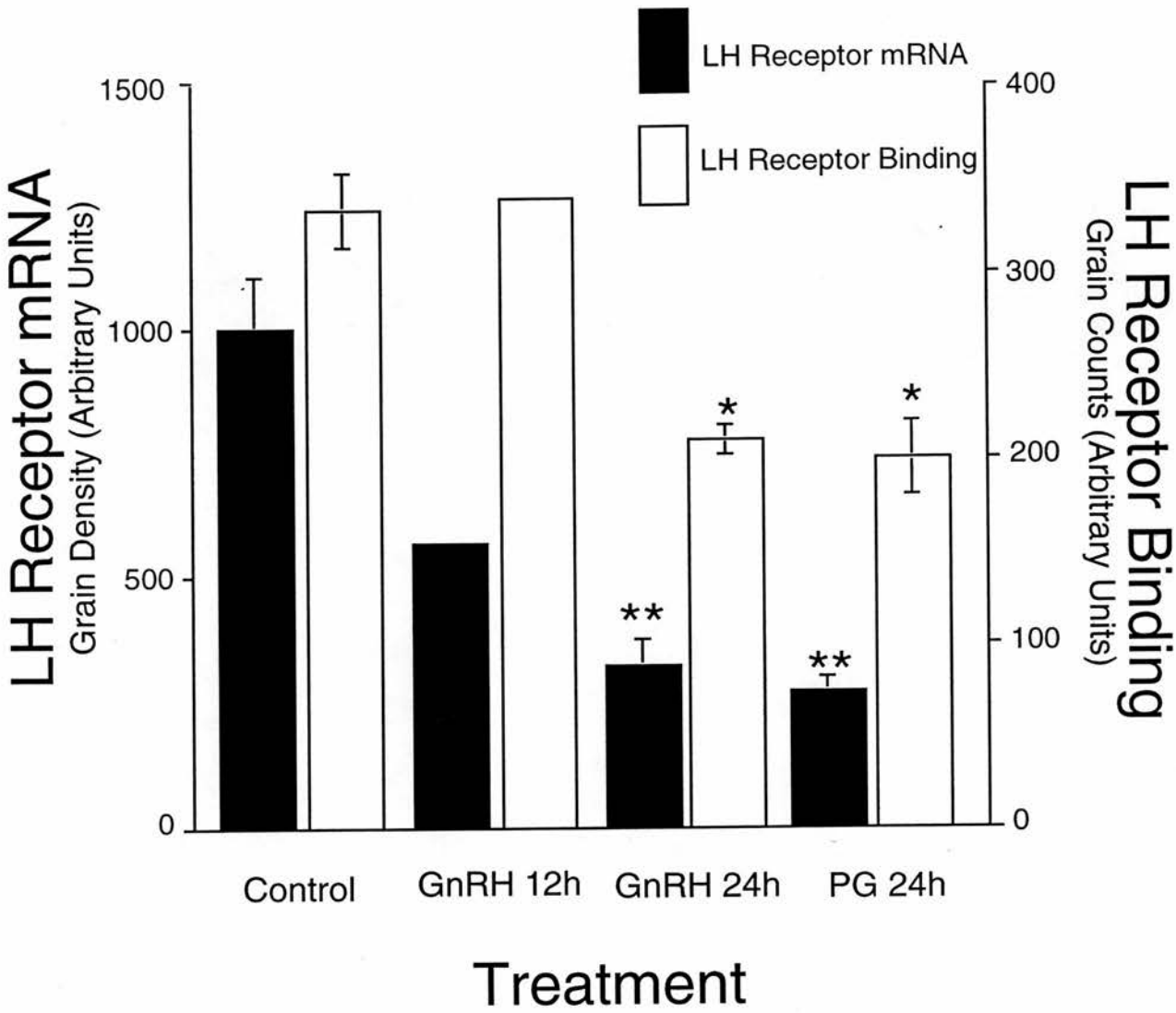


Figure 6.3

LH Receptor mRNA expression after induced luteolysis

LH receptor in marmoset corpora lutea after induced luteolysis. Grain density of LH receptor mRNA and grain counts of LH receptor binding in marmoset corpora lutea in the mid-luteal phase (control) (n=4), 12 hours after induced luteolysis with $GnRH_{ant}$ (GnRH 12h) (n=2), 24 hours after induced luteolysis with $GnRH_{ant}$ (GnRH 24h) (n=4) and 24 hours after induced luteolysis with $PGF_{2\alpha}$ (PG 24). Values are means ±S.E.M.; (*=$p<0.05$, **=$p<0.0001$ (ANOVA)).

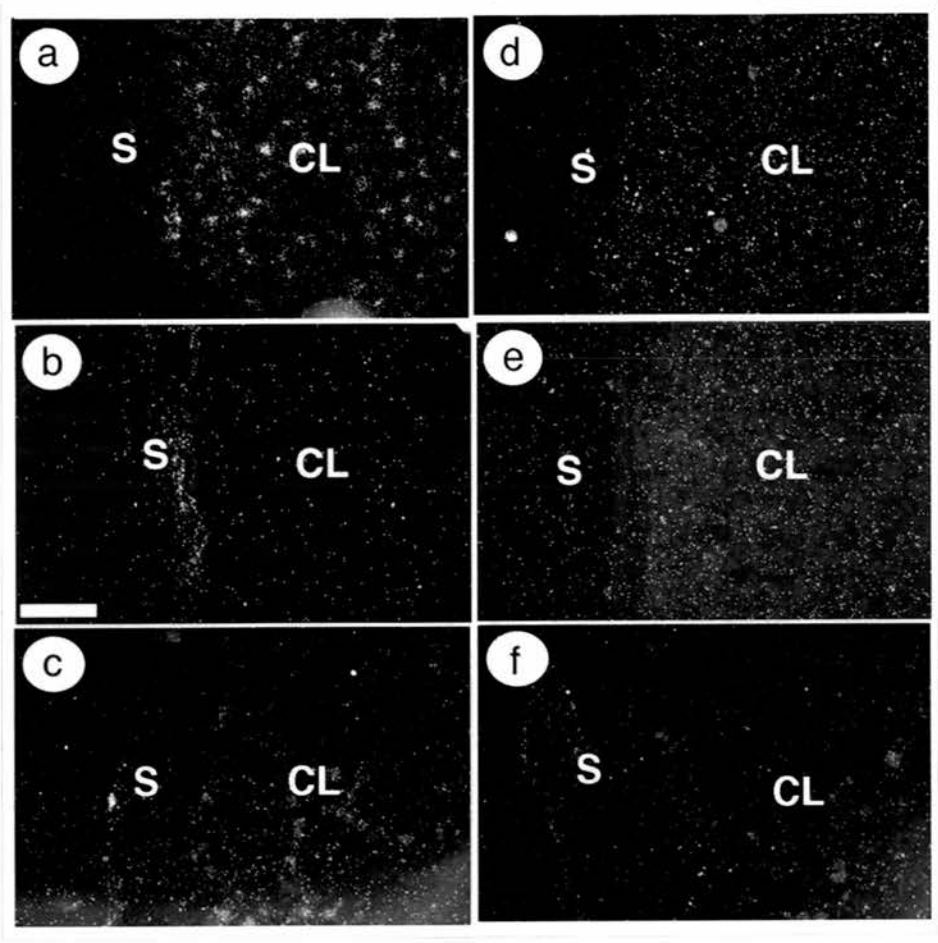


Figure 6.4

Loss of LH receptors after induced luteolysis

LH receptor in the marmoset corpus luteum 12 and 24 hours after induced luteolysis: **a)** dark-field section of a mid-luteal marmoset ovary after *in situ* hybridisation for LH receptor mRNA showing signal in the corpus luteum (CL) but not in the surrounding stroma (S); **b)** dark-field section of a marmoset ovary 12 hours and **c)** 24 hours after luteolysis was induced with $GnRH_{ant}$ after *in situ* hybridisation for LH receptor mRNA showing no signal in the corpus luteum (CL) or stroma (S); **d)** dark-field serial section of (a) showing LH binding in the corpus luteum (CL) but not the surrounding stroma (S); **e)** dark-field serial section of (b) showing persistence of LH binding in the corpus luteum (CL) and its absence from the surrounding stroma (S); f) dark-field serial section of (c) showing reduced specific LH binding in the corpus luteum (CL) and stroma (S). Scale bar = 100 μm.

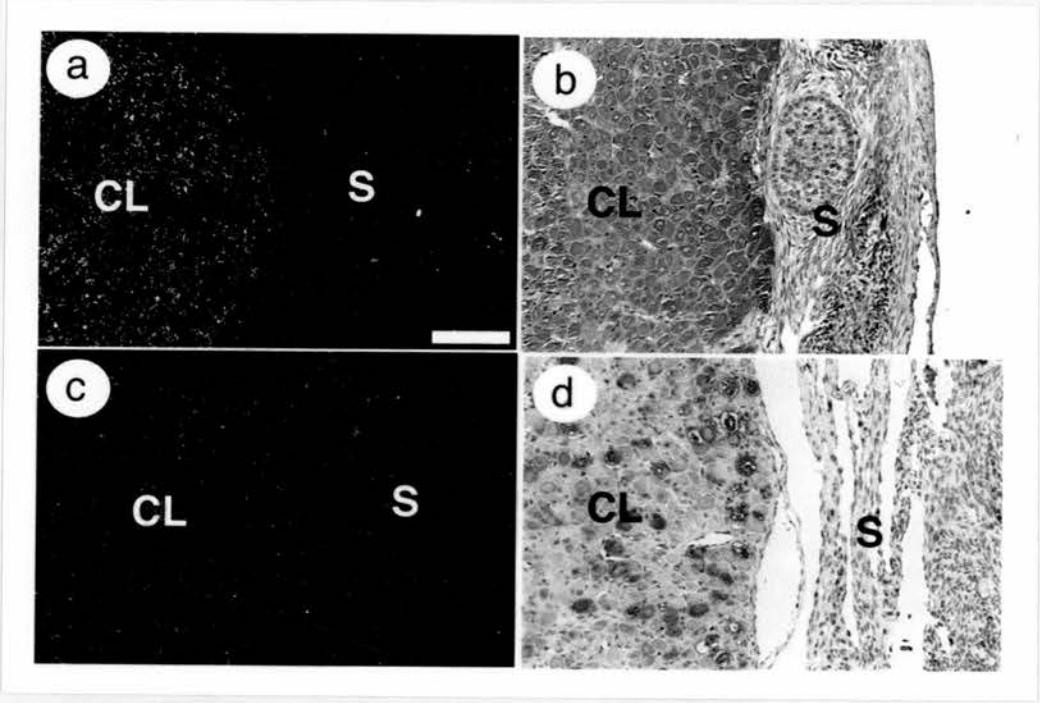


Figure 6.5

3β-HSD in the marmoset corpus luteum after induced luteolysis

3β-HSD in the marmoset corpus luteum after induced luteolysis: **a)** dark-field section of a mid-luteal marmoset ovary after *in situ* hybridisation for 3β-HSD mRNA showing signal in the corpus luteum (CL) but not the surrounding stroma (S); **b)** section of a mid-luteal marmoset ovary after immunohistochemistry for 3β-HSD showing staining of the corpus luteum (CL) but not the surrounding stroma (S); **c)** dark-field section of a marmoset ovary 24 hours after luteolysis was induced with $PGF_{2\alpha}$ after *in situ* hybridisation for 3β-HSD mRNA showing no signal in the corpus luteum (CL) and stroma (S); **d)** section of a marmoset ovary 24 hours after luteolysis was induced with $PGF_{2\alpha}$ after immunohistochemistry for 3β-HSD showing patchy staining of the corpus luteum (CL) but not the surrounding stroma (S). Scale bar = 100 μm.

increase (Ravindranath *et al.*, 1992a) or a fall (Nishimori *et al.*, 1995; Minegishi *et al.*, 1997) in luteal LH receptor mRNA during the late-luteal phase in the primate. We therefore used a well established model (Fraser *et al.*, 1996a; Fraser *et al.*, 1996b) of induced luteolysis in the primate in this follow-up study, to investigate the effect of luteolysis on LH receptor expression. It is now clear that induced luteolysis in the primate is associated with a rapid loss of LH receptors from the corpus luteum.

Induced luteolysis, has been shown to result in a fall in LH receptor expression in rodents (Bjurulf and Selstam, 1996) and ruminants (Guy *et al.*, 1995; Smith *et al.*, 1996a). In these animals, $PGF_{2\alpha}$ is an important physiological luteolysin. In rats, luteal prostaglandin increases during the last days of the luteal phase (Olofsson *et al.*, 1990), and inhibition of prostaglandin synthesis by indomethacin prolongs the natural life-span of the corpus luteum (Bjurulf *et al.*, 1994). In sheep and cows, natural luteolysis is clearly attributed to the uterine synthesis and secretion of $PGF_{2\alpha}$ (Niswender *et al.*, 1985; Auletta and Flint, 1988). In these species, it is thought that $PGF_{2\alpha}$ has a direct effect on luteal LH receptor mRNA expression (Bjurulf and Selstam, 1996; Smith *et al.*, 1996a). Although it is not clear in this study whether the reduced LH receptor mRNA is due to decreased transcription or decreased stability (Segaloff and Ascoli, 1993), it is clear that, despite different mechanisms of luteolysis in primates, (Auletta and Flint, 1988; Auletta *et al.*, 1990), the effect of $PGF_{2\alpha}$ of LH receptor mRNA levels appears to be the same.

There is evidence that locally produced $PGF_{2\alpha}$ may have a role in primate luteolysis (Auletta *et al.*, 1984b; Auletta and Flint, 1988; Behrman *et al.*, 1993). Prostaglandins are produced by the human corpus luteum and $PGF_{2\alpha}$ receptors can be detected in the human corpus luteum (Powell *et al.*, 1974; Challis *et al.*, 1976; Rao *et al.*, 1977a). Pharmacological doses of prostaglandins can reduce progesterone secretion from the primate corpus luteum (Wentz and Jones, 1973; Auletta *et al.*, 1984a). In addition, some studies have reported increased intra-luteal $PGF_{2\alpha}$ during the end of the functional human luteal phase (Shutt *et al.*, 1976; Patwardhan and Lanthier, 1980). As LH receptors cannot be detected in follicular phase primate corpora lutea (Ravindranath *et al.*, 1992a; Nishimori *et al.*, 1995; Takao *et al.*, 1997), it is clear that they disappear with functional luteolysis. Local $PGF_{2\alpha}$ may be involved in the inhibition of LH receptor expression at the end of the primate luteal phase.

The steroidogenic pathway can also be affected at other sites by $PGF_{2\alpha}$. *In vitro*, $PGF_{2\alpha}$ inhibits LH-stimulated progesterone production, and this is thought to be a post cAMP-mediated effect (Auletta and Flint, 1988; Abayasekara *et al.*, 1993; Michael *et al.*, 1994). $PGF_{2\alpha}$ is known to activate PKC (Niswender *et al.*, 1994). PKC has several effects on the steroidogenic pathway. It inhibits cholesterol transport to $P450_{scc}$ (Wiltbank *et al.*, 1993), which suggests an inhibitory effect on SCP-2 (McLean *et al.*, 1995) or StAR (Stocco and Clark, 1996). In addition, it has been shown that expression of 3β-HSD can also be inhibited *in vitro* (Hawkens *et al.*, 1993). We have shown that 3β-HSD mRNA and protein expression are inhibited during $PGF_{2\alpha}$ induced luteolysis in the primate. This confirms the multiple sites of inhibition of steroidogenesis during $PGF_{2\alpha}$-induced luteolysis are also seen in the primate.

Induced luteolysis using LH withdrawal had the same effects on luteal LH receptor and 3β-HSD expression. It has previously been shown that removal of LH support in monkeys caused a dramatic down regulation of mRNA for both $P450_{scc}$ and 3β-HSD (Ravindranath *et al.*, 1992b). Indeed, levels of 3β-HSD have been shown to decline during natural luteal regression in the primate (Doody *et al.*, 1990). We have confirmed the fall in 3β-HSD message, and shown that this is associated with a fall in LH receptor mRNA. The similarity of the effects of the disparate ways to induce luteolysis in the marmoset suggests common final pathways of action. It is not known whether LH withdrawal induces local $PGF_{2\alpha}$ formation. However, it has been shown that hCG, acting through the LH receptor (Cole *et al.*, 1973), has an inhibitory effect on luteolysis induced by $PGF_{2\alpha}$ (Auletta and Kelm, 1994). It is therefore likely that LH withdrawal, and $PGF_{2\alpha}$, activate a common final pathway to induce luteolysis.

That common pathway might be progesterone withdrawal. It is clear that acute administration of $PGF_{2\alpha}$ can inhibit progesterone synthesis in the absence of changes in the expression of steroidogenic enzymes (Michael *et al.*, 1994). Its effects on cAMP stimulation and progesterone output precede the observed decrease in mRNA levels (Khan and Rosberg, 1979; Bjurulf and Selstam, 1996). Likewise, withdrawal of LH results in rapid cessation of progesterone output (Fraser *et al.*, 1986). It is possible that progesterone itself has an autocrine role in the corpus luteum. The primate corpus luteum possesses receptors to the progesterone it produces (Chandrasekher *et al.*, 1994; Suzuki *et al.*, 1994). Recent work using trilostane to inhibit progesterone synthesis suggests that progesterone

may indeed have a major role in the function of the corpus luteum (Duffy *et al.*, 1994; Slayden *et al.*, 1994; Duffy and Stouffer, 1995). However, It is not yet clear if progesterone maintains the enzymes responsible for its production. Evidence from the rat, where $PGF_{2\alpha}$ only induces a transient decline in LH receptor and 3β-HSD mRNAs, suggests that their recovery occurs in the presence of follicular phase levels of progesterone (McLean *et al.*, 1995; Bjurulf and Selstam, 1996). The fact that we did not see this recovery, and that the rat does not appear to express luteal progesterone receptors (Parke-Sarge *et al.*, 1995) suggests that this may be a species effect. Progesterone therefore remains a potential candidate in the control of luteal function during luteolysis.

The effect of induced luteolysis on mRNA was evident before the effect on protein levels. We could detect little LH receptor and 3β-HSD mRNAs 12 hours after induced luteolysis. In sheep, Smith *et al.* (1996a) reported that this reduction was evident within six hours of induced luteolysis. We found continued LH receptor binding 12 hours after induced luteolysis and could detect some 3β-HSD protein 24 hours after induced luteolysis in the absence of mRNA. The patchy appearance of 3β-HSD we observed after induced luteolysis is similar to that seen during natural luteolysis in the monkey (Sanders and Stouffer, 1997). This time difference of effects on mRNA and protein levels was also seen in ovine corpora lutea after induced luteolysis with $PGF_{2\alpha}$ (Smith *et al.*, 1996a). This is consistent with protein having a longer turnover time than mRNA. However, as progesterone levels were at follicular levels, 12 hours after induced luteolysis, it appears that this protein is not stimulated enough to be functional at this stage. It therefore needs to be stressed that at the first time point studied (12 hours), the progesterone concentrations were already at follicular levels. It is not clear whether the decline in progesterone precedes the inhibition of LH and 3β-HSD expression, is secondary to it, or related through another common factor. It would be interesting to dissect the pathway at time points earlier than 12 hours.

Induced luteolysis in the marmoset monkey is associated with cell death and disruption of the cellular architecture (Fraser *et al.*, 1995b). Indeed it has been suggested that $PGF_{2\alpha}$ can directly cause apoptotic cell death (Sawyer *et al.*, 1990). It is possible that the fall in mRNA and protein for the LH receptor and 3β-HSD reflects a general loss of cell viability within the corpus luteum rather than being specific to functional luteolysis. This is unlikely as the loss of mRNA clearly precedes the loss of protein for both the LH receptor and 3β-HSD, and

other mRNA species are still present in the corpus luteum 12 hours after induced luteolysis (see **Chapter 11**; Duncan *et al.,* 1996b). In addition, some proteins have been shown to increase in the corpus luteum after induced luteolysis in the marmoset (Woad *et al.,* 1996). This suggests that the steroidogenic pathway is specifically and rapidly switched off during induced luteolysis.

The rapid loss of LH receptor mRNA in the corpus luteum was not seen in the thecal layers of antral follicles after induced luteolysis. This LH receptor will be equally starved of its ligand after $GnRH_{ant}$-induced luteolysis. The inhibitory effects on LH receptor and 3β-HSD mRNAs were clearly not seen in the thecal cells. This suggests that the common luteolytic pathway is not found in follicular thecal cells. It is not known whether these cells express $PGF_{2\alpha}$ receptors. In the sheep corpus luteum, $PGF_{2\alpha}$ receptors are located on the large luteal cells, and not the small luteal cells, that are thought to be of thecal origin (Fitz *et al.*, 1982). This may be one of the differences. Thecal cells of the follicle, however, may express progesterone receptors (Suzuki *et al.*, 1994). Clearly studying the differences between follicular thecal cells and luteal cells may aid understanding of the luteolytic process.

In conclusion, the rapid reduction of LH receptors and the steroidogenic enzyme, 3β-HSD, during induced luteolysis in the primate suggests an inhibition to synthesis of the components of the steroidogenic pathway as well as their function during luteolysis. It is still not clear if this is a cause or effect of low progesterone levels but it is likely to contribute to the continued inhibition of progesterone synthesis during luteolysis. The similarity of effect of $PGF_{2\alpha}$ treatment and LH withdrawal suggests a common inhibitory pathway. Dissection of this pathway may give more information about the continuing enigma of primate luteolysis.

Chapter 7

Steroidogenic enzyme expression in human corpora lutea in the absence and presence of exogenous hCG

7.1 Abstract

In a conception cycle, the expected decline in progesterone production by the human corpus luteum, during the late-luteal phase, is prevented by hCG secreted by the implanting blastocyst. This study investigated the expression of components of the synthetic pathway for progesterone in human corpora lutea in the presence and absence of hCG *in vivo*. Corpora lutea were obtained from normally cycling women at the time of hysterectomy and classified, on the basis of the urinary LH surge, as early- (n=3), mid- (n=3), or late-luteal (n=3). In addition, corpora lutea were also obtained from women who had received daily doubling doses of hCG (n=3) to 'rescue' the corpus luteum. Expression of StAR, $P450_{scc}$ and 3β-HSD were investigated by northern blotting. In addition, 3β-HSD expression was also analysed by isotopic *in situ* hybridisation and immunohistochemistry. Luteal 'rescue' with hCG was associated with the continued expression of these components of the steroidogenic pathway. In the absence of hCG, they continued to be expressed in the late-luteal phase, although their expression was more variable. The expression of 3β-HSD mRNA, however, fell during the late luteal phase ($p<0.01$). In conclusion, during luteal 'rescue', hCG acts to maintain the steroidogenic pathway. In the absence of hCG, the decline in luteal progesterone production begins in the presence of the main components of the steroidogenic pathway. It is therefore unlikely that changing expression of these components initiates this decline. However changing expression, particularly of 3β-HSD, may contribute to the continued reduction in progesterone production.

7.2 Introduction

In non-conception cycles, the human corpus luteum will undergo luteolysis, with loss of functional and structural integrity after 14 days. In the presence of hCG from the implanting blastocyst, the corpus luteum is 'rescued' and its function and structure are maintained (Behrman *et al.*, 1993). The molecular mechanisms of luteolysis and how they are prevented by exposure to hCG are still not clear in the primate.

Progesterone production by the primate corpus luteum is dependent on LH from the anterior pituitary gland (Hutchison and Zeleznik, 1984; Fraser *et al.*, 1986). LH binds to, and activates, a specific glycoprotein LH/hCG receptor present on the membranes of steroidogenic luteal cells (McNeilly *et al.*, 1980; Bramley *et al.*, 1987; Segaloff and Ascoli, 1993). The LH receptor is a G-protein-coupled receptor with a large glycosylated extracellular domain, seven transmembrane loops, and a smaller intracellular C-terminal domain (Segaloff and Ascoli, 1993). Specific ligand binding activates second messenger systems, notably cAMP (Segaloff and Ascoli, 1993). This stimulates the uptake of cholesterol into mitochondria by the action of StAR (Stocco and Clark, 1996), its conversion into pregnenolone by the enzyme $P450_{scc}$ (Simpson and Boyd, 1967) and the further conversion of pregnenolone into progesterone by 3β-HSD (Strauss and Miller, 1991).

Luteolysis can be induced by the withdrawal of LH, by inhibition of GnRH (Hutchison and Zeleznik, 1984; Fraser *et al.*, 1986). However, LH withdrawal is not the cause of luteolysis in natural cycles, as luteolysis still occurs in the presence of continued exposure to LH in the late-luteal phase (Hutchison *et al.*, 1986). This suggests that functional luteolysis is associated with an increasing block to LH action within the corpus luteum. We have therefore investigated the expression of LH/hCG receptors in human (**Chapter 5**) and monkey (**Chapter 6**) corpora lutea (Duncan *et al.*, 1996a; Duncan *et al.*, 1998a). We, and others, have shown that the primate corpus luteum continues to express LH receptors at a time when progesterone synthesis is declining (Ravindranath *et al.*, 1992a; Nishimori *et al.*, 1995; Duncan *et al.*, 1996a). Recent reports have suggested that the rate-limiting step in steroidogenesis may be the transport of cholesterol to the inner mitochondrial membrane under the action of StAR (Clark *et al.*, 1994; Stocco and Clark, 1996). In this study we therefore aimed to investigate the expression of

components of the steroidogenic pathway beyond the LH/hCG receptor, notably StAR, $P450_{scc}$ and 3β-HSD, in the human corpus luteum throughout the luteal phase and after luteal 'rescue' with hCG *in vivo* to simulate early pregnancy.

7.3 Specific Materials and Methods

7.3.1 Tissues Studied

Corpora lutea were enucleated at the time of hysterectomy in 12 women undergoing surgery for benign conditions (**2.2**) and dated on the basis of serial urinary LH measurements (**2.2.4**). Three corpora lutea were classified as early-luteal, three as mid-luteal and three as late-luteal (**2.2.4**). Three corpora lutea were collected from women after luteal 'rescue' with exogenous hCG (**2.2.2**). One piece of each corpus luteum was fixed in 4% paraformaldehyde and embedded in paraffin wax (**2.2.3**) for subsequent immunohistochemistry, another piece was frozen in embedding medium for the preparation of frozen sections (**2.2.3**) and a third piece was stored at -70 °C for subsequent RNA extraction (**2.2.3**). An endometrial biopsy was also fixed in paraformaldehyde and processed into paraffin wax for luteal phase dating by tissue morphometry (**3.2.8**).

7.3.2 Cloning of StAR from the Human Corpus Luteum

Total cellular RNA was extracted from a mid-luteal human corpus luteum by the method of Chomczynski and Sacchi (1987) and reverse transcribed into cDNA using a commercial kit (Promega). Specific oligonucleotide primers for StAR were synthesised by Oswell DNA Services (Southampton, Hants, UK). The primer sequences, 5'-AACCAGGAAGGCTGGAA-3' and 5'-CCATGCAGGTG-GGGCCGTGTTCAGC-3' (Clark *et al.*, 1994) were used to amplify the expected 400 bp fragment of StAR cDNA using the PCR conditions as described previously (Clark *et al.*, 1994). This was cloned into a plasmid PCR II vector using a commercial kit (TA Cloning Kit; Invitrogen, NV Leek, Netherlands) and sequenced using a *Taq* di-deoxy termination cycle sequencing kit (Applied Biosystems, Warrington, Cheshire, UK) using an automatic sequencer (373A; Applied Biosystems). Data were analysed using a commercial computer program (GeneJockey II; Biosoft, Cambridge, Cambs, UK).

7.3.3 Northern Blotting

Twenty micrograms of total cellular RNA was denatured, electrophoresed in a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane and fixed by u.v. cross-linkage (**3.6.1**). The membranes were then pre-hybridised for 5 h in hybridisation buffer (**3.6.2**). The cDNA probes (**3.1.3**) were labelled with 50 μCi ^{32}P dCTP by the random priming method (**3.5.5**) and added to the hybridisation buffer for 20 h at 65 °C (**3.6.2**). The membranes were washed (**3.6.3**), laid down to a phosphor screen for 48 h and visualised using a phosphorimager computer (**3.6.3**). The blots were then stripped and then re-probed for 18S RNA (**3.6.4**). The molecular size of the detected transcripts was determined by running RNA markers in an adjacent lane (**3.6.1**).

7.3.4 *In situ* Hybridisation

Isotopic *in situ* hybridisation was performed on frozen sections using ^{35}S-labelled riboprobes (**3.7**). The antisense 3β-HSD riboprobe (**3.1.3**) was generated by T7 RNA polymerase (**3.7.2**). Frozen sections were quickly thawed and fixed in 4% paraformaldehyde (**3.7.3**). The slides were then washed, acetylated, dehydrated and dried under vacuum (**3.7.3**). One hundred microlitres of hybridisation buffer containing 1 x 10^6 c.p.m. radiolabelled probe was added to each section (**3.7.4**). The slides were covered with a hydrophobic coverslip and incubated overnight at 55 °C in a moist chamber (**3.7.4**). The coverslips were then washed off and the slides were treated with RNAse A (**3.7.5**). The sections were then washed in increasingly stringent conditions, dehydrated and allowed to dry (**3.7.5**). They were dipped in photographic emulsion (**3.7.6**) and stored at 4 °C for 18 days in the dark. After developing and fixing (**3.7.6**), the slides were washed in water, counterstained with haematoxylin and mounted (**3.2.8**). The sections were viewed and photographed under dark-field illumination. The localisation of the grains was determined by reference to the section viewed under bright-field.

7.3.5 Immunohistochemistry

Paraffin wax sections on poly-L-lysine-coated slides (**3.2.1**) were de-waxed and rehydrated (**3.2.2**). After the endogenous peroxidase activity was blocked (**3.2.5**), non-specific antibody binding was blocked with 20% NGS in TBS with 4% BSA (**3.2.6**). Sections were incubated overnight at 4 °C with the polyclonal rabbit anti-human 3β-HSD antisera (**3.1.2**) diluted 1:1000 in 20% NGS in TBS (**3.2.6**).

Specific antibody binding was detected with biotinylated goat anti-rabbit immunoglobulins diluted 1:500 in TBS (**3.2.6**), and visualised with AB-HRP using DAB to give a stable brown end product (**3.2.7**). The sections were counterstained with haematoxylin, dehydrated and mounted (**3.2.8**). Polyclonal rabbit IgG at the same antibody concentration was used in place of the primary antibody, in serial sections, as a negative control (**3.2.9**).

7.3.6 Analysis of Results

Northern blot band intensity was measured using the phosphorimager computer. To correct for minor differences in loading, the ratio of the relative band intensity to the 18S band intensity was used for data analysis. One-way ANOVA was used to investigate differences in expression throughout the luteal phase. The early-luteal corpora lutea were compared to the mid and late-luteal corpora lutea using an unpaired t-test. A commercial software package was used for statistical analysis (StatView 4.0). Immunohistochemical staining for 3β-HSD was assessed in sections from the same run performed under carefully controlled conditions by an observer blinded to tissue identity. This was repeated two weeks later to confirm consistency of scoring. The staining intensity in the granulosa-lutein cell layer was graded as absent (-), faint (+), moderate (++), intense (+++) or very intense (++++) for each section. Where the sections differed between each scoring session an intermediate value was given.

7.4 Results

7.4.1 Plasma Progesterone Concentrations

The classification of the corpora lutea by serial urinary LH measurement agreed with the luteal-phase dating of endometrial biopsies using the method of Li *et al.* (1988). The plasma progesterone concentrations were 35.3 ±9.8 nmol/l in the early-luteal samples, 41.0 ±9.9 nmol/l in the mid-luteal samples and 19.2 ±12.9 nmol/l in the late-luteal samples. After luteal 'rescue' by exogenous hCG the plasma progesterone concentrations had increased to 52.6 ±1.5 nmol/l.

7.4.2 Cloning of Human StAR

The specific oligonucleotide primers for StAR amplified the expected 400 bp fragment (Clark *et al.*, 1994) from cDNA obtained from a mid-luteal human corpus luteum. When this fragment was sequenced, the sequence was 97% identical to the sequence for human StAR in the gene sequence databases. This StAR cDNA sequence was used to study the expression of StAR mRNA in the human corpus luteum.

7.4.3 Expression of Steroidogenic Enzymes in Human Corpora Lutea

Specific mRNA transcripts for StAR, $P450_{scc}$ and 3β-HSD could be detected in corpora lutea from different stages of the luteal phase and after luteal 'rescue' with exogenous hCG (Fig. 7.1). Two major StAR mRNA transcripts of 1.7 kb and 4.8 kb were detected. These are consistent with the size of human StAR transcripts already published (Kiriakidou *et al.*, 1996). Major transcripts of 2.0 kb and 1.7 kb were detected in corpora lutea after northern blotting for $P450_{scc}$ and 3β-HSD respectively (Fig. 7.1) These are consistent with the expected size of the mRNAs for these steroidogenic enzymes in the primate (Doody *et al.*, 1990; Bassett *et al.*, 1991).

7.4.4 Changes in Steroidogenic Enzyme Expression in the Luteal Phase

As can be seen in Fig. 7.1, expression of the steroidogenic enzymes did not appear to be constant throughout the luteal phase. In the late-luteal phase band intensity was more variable than during other stages of the luteal phase. Band intensity was measured and corrected for 18S RNA expression for each steroidogenic enzyme (Fig. 7.2). It can be seen that expression of enzymes tended to be lower in the late-luteal phase. However, this trend did not reach statistical significance for any of the enzymes. However, when the mid- and late-luteal phases were combined, the expression of 3β-HSD was markedly lower ($p<0.01$) than in the early-luteal phase or after luteal 'rescue' with hCG. The most marked change in steroidogenic enzyme expression throughout the luteal phase was that of 3β-HSD.

7.4.5 Localisation and Expression of 3β-HSD in Human Corpora Lutea

As northern blotting revealed that 3β-HSD changed most during the luteal phase, its expression and localisation were further investigated by isotopic *in situ*

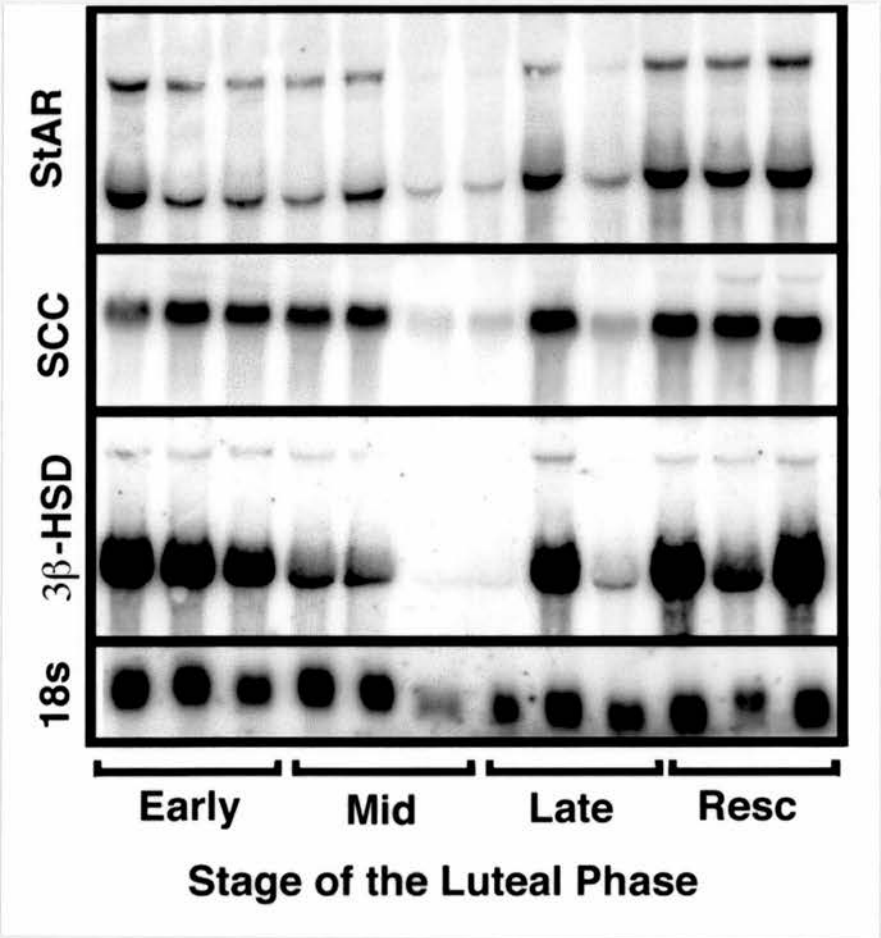


Figure 7.1

Expression of the steroidogenic pathway in corpora lutea

Composite picture of northern blots for StAR, $P450_{scc}$ and 3β-HSD mRNA in human corpora lutea at different stages of the luteal phase. Specific hybridisation bands are shown in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late-luteal (LH+11 to LH+14) phases and after luteal 'rescue' with exogenous hCG (hCGx5 to hCGx7). The intensity of the 18S RNA band is shown, after specific oligonucleotide hybridisation, to confirm equal mRNA loading between lanes. Unfortunately, there has been some under-loading of one of the mid-luteal samples in these blots.

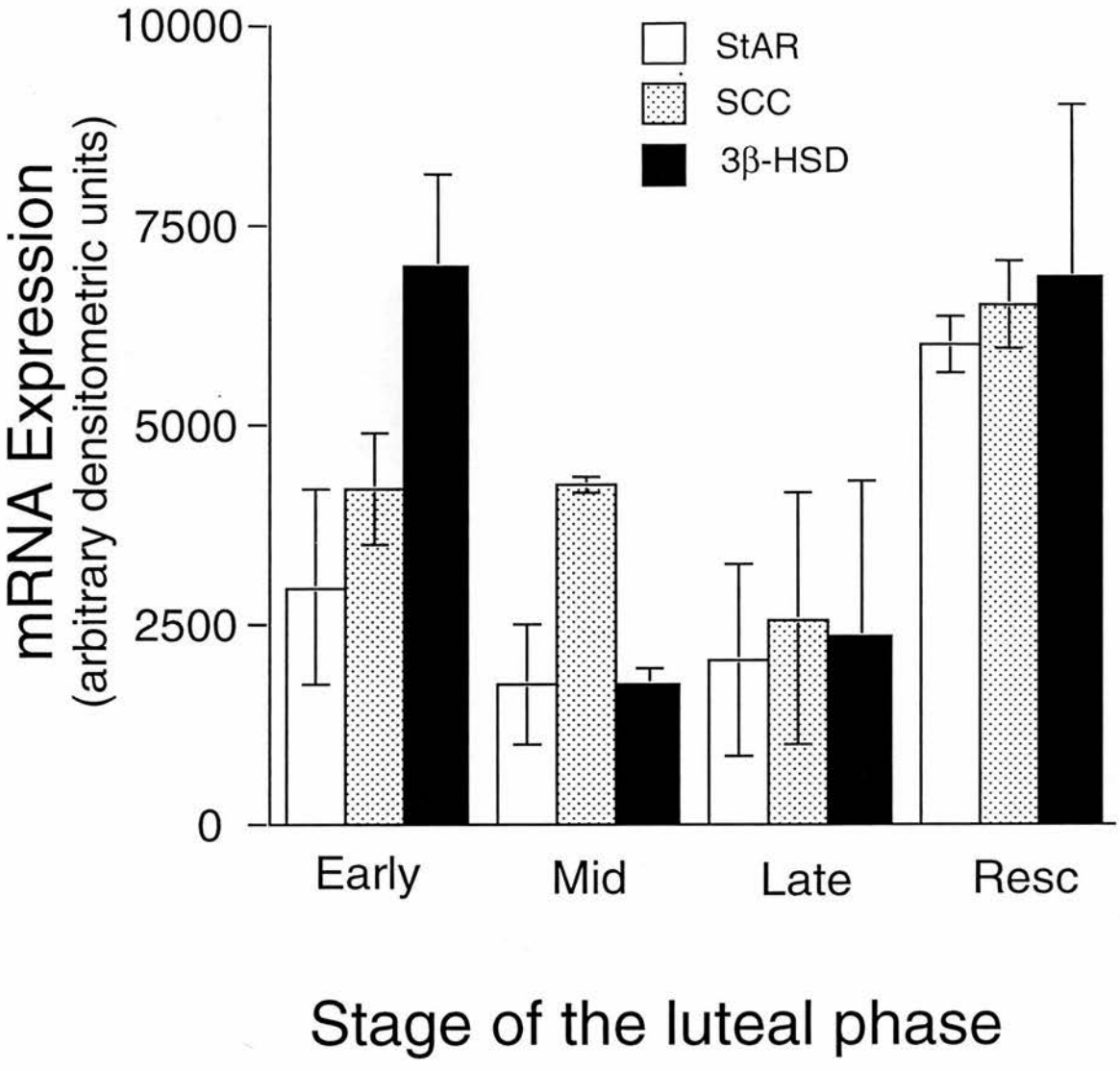


Figure 7.2

Quantification of steroidogenic enzyme expression in human corpora lutea

Expression of mRNA for steroidogenic factors as measured by corrected hybridisation band intensity after northern blotting in human corpora lutea at different stages of the luteal phase. The mean band intensities for the 1.7 kb StAR, the 2.0 kb $P450_{scc}$ and the 1.7 kb 3β-HSD mRNA transcripts are shown ±S.E.M., in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late-luteal (LH+11 to LH+14) phases and after luteal 'rescue' with exogenous hCG (hCGx5 to hCGx7) (n=3 per group). There were no significant differences across the luteal phase (ANOVA). When the early-luteal samples were compared with the mid- and late-luteal samples, the expression of 3β-HSD was found to decrease from the early-luteal phase ($p<0.01$; t-test).

hybridisation and immunohistochemistry. Messenger RNA could be detected by *in situ* hybridisation in corpora lutea at all stages of the luteal phase. However, during the late-luteal phase, detection of 3β-HSD mRNA was variable. The hybridisation signal at the beginning of the late-luteal phase (Fig. 7.3*a*) was similar to that seen in the mid-luteal phase. However, towards the end of the late-luteal phase much less 3β-HSD mRNA could be detected by *in situ* hybridisation (Fig. 7.3*c*). In contrast, 3β-HSD protein, as detected by immunohistochemistry, could be detected throughout the late-luteal phase (Fig. 7.3*b,d*). When 3β-HSD immunostaining was analysed, the intensity did not vary across the luteal phase or after luteal 'rescue' with exogenous hCG (Fig. 7.4).

7.5 Discussion

We have previously reported the expression of LH/hCG receptors in human corpora lutea throughout the luteal phase and after luteal 'rescue' with hCG (Duncan *et al.*, 1996a). We found that LH/hCG receptor mRNA and binding activity were retained in corpora lutea during the late-luteal phase when progesterone production is falling. There is a decline in LH secretion during the luteal phase (Ellinwood *et al.*, 1984), but this is not wholly responsible for the falling luteal progesterone production (Hutchison *et al.*, 1986). The reasons for reduced progesterone synthesis in the late-luteal phase in the presence of LH and LH receptors are therefore not yet clear. The stage of steroidogenesis which is generally thought to be rate-limiting is the conversion of cholesterol to pregnenolone (Strauss and Miller, 1991; Stocco and Clark, 1996). This stage involves the actions of both StAR (Stocco and Clark, 1996) and $P450_{scc}$ (Simpson and Boyd, 1967). As the tools are now available to investigate StAR expression at a molecular level, in this follow-up study, we looked at its expression during the luteal phase and compared its expression with that of the steroidogenic enzymes involved in progesterone synthesis, $P450_{ssc}$ and 3β-HSD.

We have cloned StAR from the human corpus luteum and shown it to be expressed throughout the luteal phase and after luteal 'rescue' with exogenous hCG. Since it was first characterised at a molecular level in 1994 (Clark *et al.*, 1994), it has become increasingly clear that StAR has a fundamental role in the control of steroidogenesis (Lin *et al.*, 1995; King *et al.*, 1995). It has been shown to be involved in steroidogenesis in ovine (Juengel *et al.*, 1995) and bovine

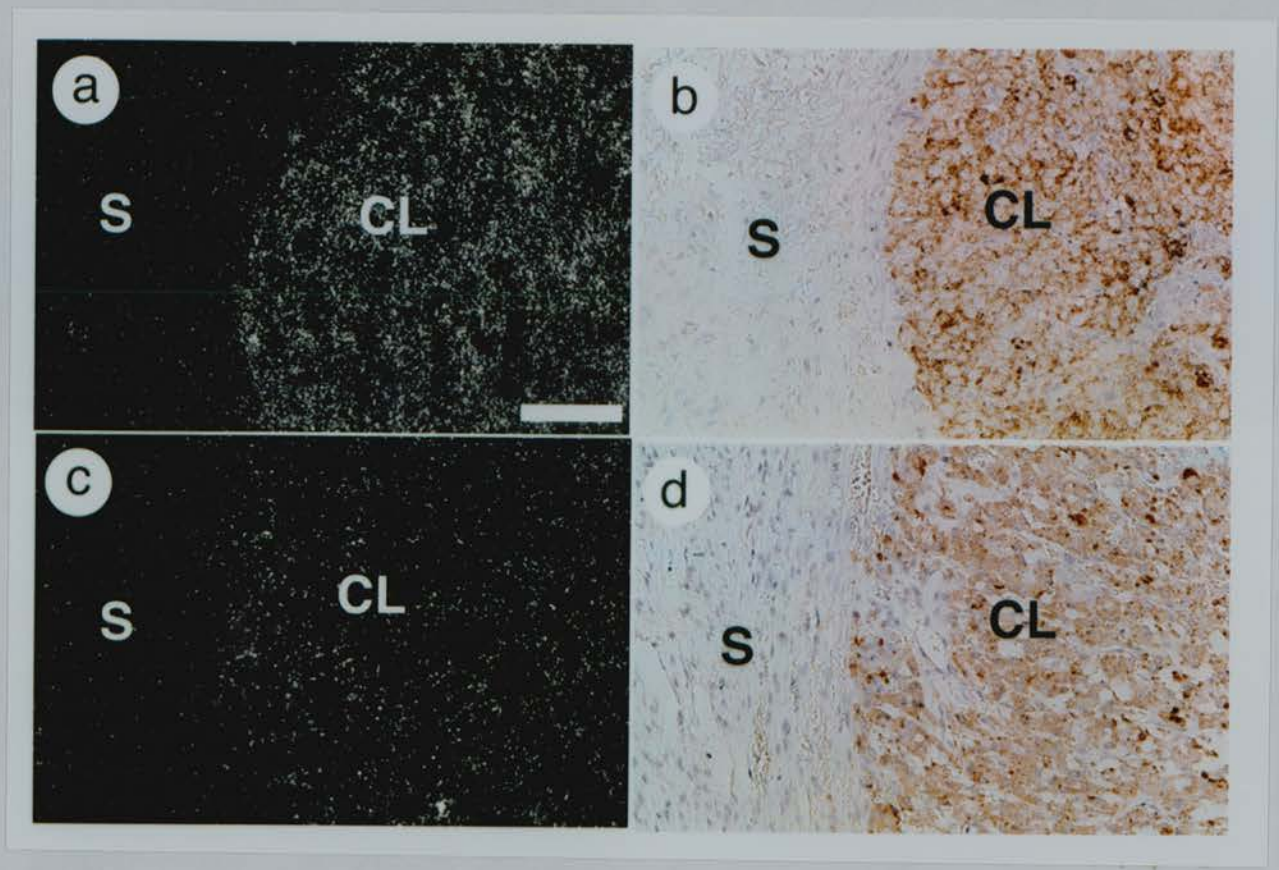


Figure 7.3

3β-HSD mRNA and protein in late-luteal corpora lutea

Expression and immunolocalisation of 3β-HSD in the late-luteal human corpus luteum: **a)** dark-field view of a corpus luteum from LH+11 after isotopic *in situ* hybridisation for 3β-HSD mRNA showing expression in the corpus luteum (CL) but not in the surrounding stroma (S); **b)** the same corpus luteum as (a) after immunohistochemistry for 3β-HSD protein showing specific staining in the corpus luteum (CL) and not in the surrounding stroma (S); **c)** dark-field view of a corpus luteum from LH+14 after isotopic *in situ* hybridisation for 3β-HSD mRNA showing no expression in the stroma (S) and little expression in the corpus luteum (CL); **d)** the same corpus luteum as (c) after immunohistochemistry for 3β-HSD protein showing continued specific immunostaining in the corpus luteum (CL) with no staining in the surrounding stroma (S). Scale bar =100 μm.

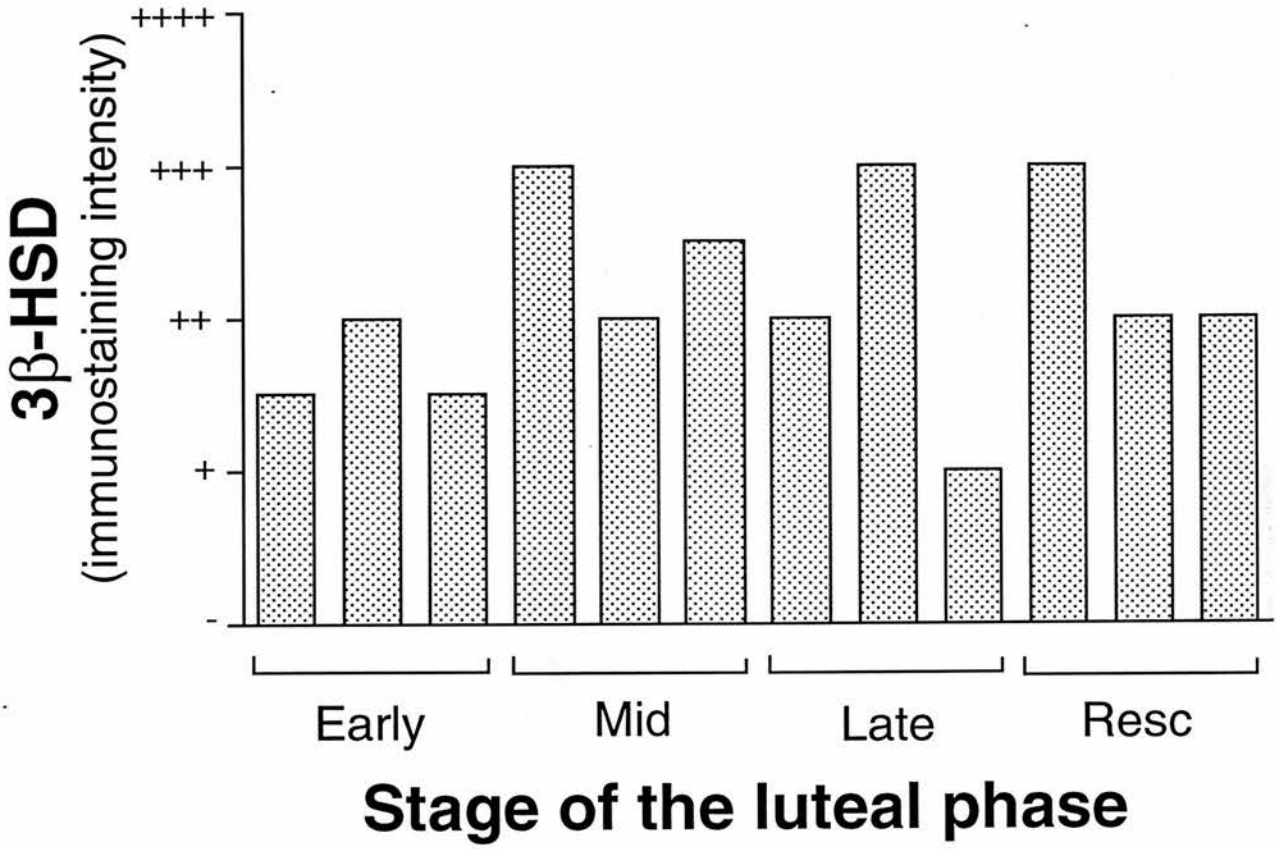


Figure 7.4

3β-HSD immunostaining in human corpora lutea

Intensity of immunostaining for 3β-HSD in the human corpus luteum at different stages of the luteal phase. Staining intensity was graded as absent (-), faint (+), moderate (++), intense (+++) or very intense (++++) by an observer blinded to tissue identity. There is little variation in staining intensity in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late-luteal (LH+11 to LH+14) phases or after luteal 'rescue' with exogenous hCG (hCGx5 to hCGx7).

(Hartung *et al.*, 1995) corpora lutea. Recently, Kiriakidou *et al.* (1996) reported the expression of StAR in the human ovary. We have confirmed that StAR is expressed by the human corpus luteum during the functional luteal phase after luteal 'rescue' in simulated early pregnancy. This suggests that expression of StAR may be a major factor in the control of luteal steroidogenesis.

StAR is a good candidate molecule to explain how progesterone production falls in the late-luteal phase and is increased by hCG during luteal 'rescue'. Expression of StAR has been shown to be regulated by LH and cAMP in granulosa-luteal cells (Sugawara *et al.*, 1995; Kiriakidou *et al.*, 1996), and it is the expression of StAR that is now thought to be the major rate limiting step in the steroidogenic pathway (Stocco and Clark, 1996). However, we have found that StAR expression continues across the luteal phase in a similar fashion to $P450_{scc}$ and the LH receptor (Duncan *et al.*, 1996a). Clearly, the elements of the steroidogenic pathway must decline in the late-luteal phase, as LH receptor and steroidogenic enzyme expression is absent from corpora lutea collected during menstruation (Doody *et al.*, 1990; Bassett *et al.*, 1991; Ravindranath *et al.*, 1992a; Nishimori *et al.*, 1995). In our study, there was more heterogeneity of expression in the late-luteal phase but this did not reach statistical significance. It appears that the fall in progesterone production occurs in the continued expression of the main elements of the steroidogenic pathway including StAR. It is unlikely that differential regulation of StAR expression initiates functional luteolysis.

While expression of StAR seems to vary in a similar fashion to $P450_{scc}$ and LH receptors (Duncan *et al.*, 1996a), the changes in expression of 3β-HSD were more marked. Of the components of the steroidogenic pathway studied, we found the expression of 3β-HSD mRNA to vary most throughout the luteal phase. Previous studies of steroidogenic enzyme expression in primate corpora lutea have shown similar results. Doody *et al.* (1990) reported expression of $P450_{scc}$ and 3β-HSD in a mid-luteal and a late-luteal human corpus luteum. Whereas $P450_{scc}$ was easily detected in the late-luteal phase, this was not true of 3β-HSD. In a larger study of the primate corpus luteum, Bassett *et al.* (1991) found no change in the expression of $P450_{scc}$ mRNA but a marked reduction in 3β-HSD mRNA during the functional luteal phase. Although 3β-HSD is not classically thought to be rate-limiting in steroidogenesis (Strauss and Miller, 1991), its expression appears to be more tightly regulated than other steroidogenic components in the primate corpus luteum.

We found the steroidogenic components to be expressed in high levels in the early-luteal corpus luteum when progesterone production is increasing. This agrees with previous studies on the primate corpus luteum (Doody *et al.*, 1990; Bassett *et al.*, 1991). It is possible that the increase in progesterone production in the early-luteal phase is due to the increasing translation of this mRNA into functional enzymes. However, we could immunolocalise 3β-HSD at this stage, and other groups have reported no differences in immunodetectable steroidogenic enzymes in the early-luteal phase (Suzuki *et al.*, 1993; Conley *et al.*, 1995; Sanders and Stouffer, 1997). The likely explanation for the apparent difference in steroidogenic capacity in the early-luteal phase and the circulating progesterone levels is substrate availability (Carr *et al.*, 1982; Bassett *et al.*, 1991).

It is uncertain whether the changes in 3β-HSD expression are involved in the initiation of functional luteolysis. We, and others (Bassett *et al.*, 1991), have provided evidence that the fall in 3β-HSD mRNA expression begins in the mid-luteal phase at a time of maximal progesterone output. *In vivo*, maximal stimulation of progesterone production from luteal cells occurs in the early-luteal phase and declines throughout the rest of the luteal phase (Fisch *et al.*, 1990). Although there may be changes in the distribution of 3β-HSD immunostaining in the late-luteal stage (Sanders and Stouffer, 1997), we and others have shown that 3β-HSD protein continues to be present, and presumably functional, throughout the luteal phase (Suzuki *et al.*, 1993; Hild-Petito and Fazleabas, 1997). Inhibition of 3β-HSD, using trilostane, inhibits progesterone function but the non-steroidogenic functions of the corpus luteum, such as relaxin production continue normally (Duffy *et al.*, 1994; Duffy *et al.*, 1995). Other factors must be involved in controlling the lifespan of the corpus luteum. A fall in 3β-HSD activity does not appear to be able to initiate normal luteolysis by itself.

The mechanism for the apparent differential control of 3β-HSD and $P450_{scc}$ expression is not known. It remains possible that it is a function of mRNA stability and different half-lives of mRNA species. There is some preliminary evidence for differential control of expression of these enzymes. In rats (Oonk *et al.*, 1989), and humans (Voultilainen *et al.*, 1986) it appears that after the LH surge, expression of $P450_{scc}$ is constitutive and not augmented by gonadotrophin stimulation. In contrast, gonadotrophins have been shown to have a slight stimulatory effect of 3β-HSD expression *in vitro* (Chedrese *et al.*, 1990). However, in the primate corpus luteum, Ravindranath *et al.* (1992b) reported

similarities in the control of $P450_{scc}$ and 3β-HSD expression by showing LH is mandatory for the continued expression of both. Further work is required to study the control of 3β-HSD expression in the corpus luteum.

All elements of the steroidogenic pathway were maintained in the 'rescued' corpus luteum of simulated early pregnancy. In granulosa cells, the LH surge initiates the expression of $P450_{scc}$ and 3β-HSD (Strauss and Miller, 1991). Recently, it has been shown that granulosa cell StAR expression is also initiated by LH at the time of the LH surge (Kiriakidou *et al.*, 1996). Administration of $GnRH_{ant}$ in the mid-luteal phase clearly shows that LH is required for the continued expression of these enzymes in the corpus luteum (Ravindranath *et al.*, 1992b). During luteal 'rescue', hCG acts through the LH receptor (Cole *et al.*, 1973) to prevent luteolysis and maintain progesterone production. It appears that LH/hCG has a stimulatory effect on the expression of the key elements of the steroidogenic pathway. One of the effects of hCG in early pregnancy appears to be the facilitation of the expression of the enzymes responsible for progesterone synthesis.

The causes of the fall in the expression of luteal steroidogenic enzymes towards menstruation is not clear. Expression is variable in the late-luteal phase and absent from the corpus luteum after menstruation (Bassett *et al.*, 1991; Suzuki *et al.*, 1993; Sanders and Stouffer, 1997). It appears that, at the beginning of the late-luteal phase, when progesterone production is falling, elements of the steroidogenic pathway are still being expressed. At the end of the luteal phase, expression of these elements, including LH receptors, appears to be reduced (Bassett *et al.*, 1991; Ravindranath *et al.*, 1992b; Suzuki *et al.*, 1993; Nishimori *et al.*, 1995). As the production of progesterone, and the maintenance of steroidogenic enzyme expression, is dependant on LH (Hutchison and Zeleznik, 1984; Fraser *et al.*, 1986; Ravindranath *et al.*, 1992b), it is possible that, in the late-luteal phase, LH action is being diluted at the level of the LH receptor (Zeleznik and Hillier, 1996). As LH receptors are still present (Ravindranath *et al.*, 1992a; Duncan *et al.*, 1996a), this effect may be at the level of coupling to second messenger systems. Studies of the LH receptor have shown that uncoupling does occur in some circumstances (Segaloff and Ascoli, 1993). It is likely that the continued stimulation of the steroidogenic enzymes also stimulates their continued expression.

In conclusion, the molecular mechanisms of the initiation of functional luteolysis are still unknown. It is likely that a reduction in expression of the components of

the steroidogenic pathway, particularly 3β-HSD, is involved in the continued fall in progesterone production premenstrually. It is, however, unlikely that changes in their expression initiates the initial reduction in progesterone production. In the presence of logarithmically increasing concentrations of hCG, in early pregnancy, the steroidogenic pathway is maintained, facilitating the continuing luteal synthesis of progesterone. In the late-luteal phase, the fall in progesterone production appears to occur in the presence of the major components of the steroidogenic pathway, including StAR. The fall in progesterone production is then associated with alterations in steroidogenic enzyme expression, particularly 3β-HSD. The mechanisms of the initial drop in progesterone production, and why the expression of 3β-HSD appears to vary most throughout the luteal phase remain unclear. It appears that further studies on luteal steroidogenesis in the primate, focusing on the stimulation of second messenger signals in response to LH receptor ligand binding, are required.

Chapter 8

The human corpus luteum continues to expresses the progesterone receptor during maternal recognition of pregnancy

8.1 Abstract

It has been suggested that progesterone may have an autocrine role in the maintenance of luteal function. This study aimed to localise progesterone receptors in the human corpus luteum and endometrium, and to investigate the effect of luteal 'rescue' with exogenous hCG. Corpora lutea and endometrial biopsies were collected from women undergoing hysterectomy throughout the luteal phase and after luteal 'rescue' with exogenous hCG. Progesterone receptors were detected by immunohistochemistry using monoclonal antibodies which recognised both 'A' and 'B' isoforms, or solely the 'B' isoform, of the receptor. Steroidogenic cells were identified by immunohistochemistry for 3β-HSD. In the endometrium, the strong glandular epithelial staining present in early-luteal tissue did not persist beyond this stage ($p<0.005$). Stromal staining persisted throughout the luteal phase. Progesterone receptors were much more difficult to immunolocalise in the corpus luteum. They could be localised to the steroidogenic cells and some cells in the surrounding stroma. The localisation or intensity of immunostaining did not change across the luteal phase or after luteal 'rescue' with hCG, or when the antibody recognising the 'B' isoform was used. In conclusion, the corpus luteum continues to express progesterone receptors, particularly the 'B' isoform, in the presence of increasing progesterone concentrations during luteal 'rescue'. The low levels of luteal progesterone receptors and the lack of change in relation to luteal function, does not add support to the hypothesis that progesterone has a major autocrine role in the human corpus luteum.

8.2 Introduction

The molecular mechanisms of luteolysis and luteal 'rescue' in the human corpus luteum remain unclear (Behrman *et al.*, 1993). It is clear, however, that the major function of the corpus luteum is the secretion of progesterone, to prepare the endometrium for blastocyst implantation, and to support early pregnancy (Knobil, 1973; Behrman *et al.*, 1993; Zeleznik and Fairchild Benyo, 1994). Progesterone is synthesised from cholesterol by the action of steroidogenic enzymes (Strauss *et al.*, 1981), under the control of pituitary LH (Hutchison and Zeleznik, 1984), and secreted by the steroidogenic cells of the corpus luteum. Progesterone acts on its target tissues through a specific nuclear hormone receptor (Grody *et al.*, 1982). To date, three isoforms of the nuclear progesterone receptor have been described 'A', 'B' (Horwitz *et al.*, 1985; Kastner *et al.*, 1990) and 'C' (Wei *et al.*, 1990). The 'A' form of the receptor is a slightly truncated version of the 'B' form, and the 'C' form is more truncated still (Wei *et al.*, 1990). Although not fully characterised, it has become apparent that these different forms of the receptor may have different functional properties (Kastner *et al.*, 1990; Vegeto *et al.*, 1993; Tung *et al.*, 1993; Graham and Clarke, 1997).

The main site of progesterone action is the uterine endometrium (Lessey *et al.*, 1988; Press *et al.*, 1988) and myometrium (Kawaguchi *et al.*, 1991), but there are many extra-uterine sites of action. Progesterone receptors have been localised in the brain (Press and Greene, 1988), blood vessels (Perrot-Applanat *et al.*, 1994), breast (Clark *et al.*, 1983), lung (Press and Greene, 1988) and luteinising granulosa cells (Greenberg *et al.*, 1990). However, of particular interest is the detection of progesterone receptors in the corpus luteum itself. Specific nuclear progesterone receptors have been detected in the corpus luteum of non-human primates (Hild-Petito *et al.*, 1988) and women (Iwai *et al.*, 1990; Horie *et al.*, 1992; Suzuki *et al.*, 1994). However, other species, such as the rat, do not appear to express luteal progesterone receptors (Park-Sarge *et al.*, 1995). The finding of specific progesterone receptors in progesterone-producing cells gives new support to a theory, put forward many years ago, that progesterone is involved in controlling its own synthesis and secretion (Rothchild, 1981; Rothchild, 1996).

We postulated that if progesterone had a significant autocrine effect in the corpus luteum, the expression of luteal progesterone receptors may vary throughout its functional lifespan. We investigated the expression and localisation of

progesterone receptors in the human corpus luteum by immunohistochemistry throughout the luteal phase and after luteal 'rescue' with exogenous hCG to mimic the hormonal changes of early pregnancy. We identified steroidogenic cells in serial sections using an antibody to the steroidogenic enzyme 3β-HSD. In addition, we investigated the nature of progesterone receptor by looking specifically for the 'B' isoform. The expression and localisation of the progesterone receptor in a known, hormonally-regulated, progesterone target site, the endometrium, was also studied throughout the luteal phase and after luteal 'rescue'.

8.3 Specific Materials and Methods

8.3.1 Tissues Studied

Corpora lutea and endometrial biopsies from 25 women were studied (**2.2**). Part of each corpus luteum and the endometrial biopsy was fixed for 24 hours in 4% paraformaldehyde and embedded in paraffin wax (**2.2.3**). In addition, part of the corpus luteum was frozen in embedding medium for the preparation of frozen sections (**2.2.3**). Each tissue was classified according to the date of the urinary LH surge (**2.2.4**). Six samples were classified as early-luteal, six as mid-luteal, six as late-luteal and six were classified as luteal 'rescue' (**2.2.4**). One sample was classified as periovulatory and consisted primarily of luteinised granulosa cells. Haematoxylin and eosin stained sections of each corpus luteum and endometrial biopsy were prepared (**3.2.8**).

8.3.2 Immunohistochemistry

Immunohistochemistry was initially performed on paraformaldehyde-fixed sections of corpus luteum and endometrium (**3.2.2**). Sections (5 μm), cut onto poly-L-lysine-coated slides (**3.2.1**), were used. As preliminary experiments showed that microwave antigen retrieval was required, this was performed as described previously (**3.2.4**). Immunohistochemistry was performed using NRS to block non-specific binding (**3.2.6**). The mouse anti-human progesterone receptor antibody (Battifora and Mehta, 1993) (**3.1.2**) was used at a dilution of 1:20 in TBS. This antibody recognises both the 'A' isoform and the 'B' isoform of the receptor and has no cross-reactivity with other steroid-hormone receptors.

Specific antibody binding was visualised using biotinylated rabbit anti-mouse immunoglobulins and an AB-AP detection system (**3.2.6**). As negative controls, the primary antibody was replaced by mouse immunoglobulins, at the same concentration, in serial sections (**3.2.9**). Colouration was achieved by incubation with NBT (**3.2.7**). This gives a stable blue end-product. Care was taken to ensure that all slides were processed identically and were exposed to each step of the protocol for exactly the same time, under the same conditions. The sections were not counterstained with haematoxylin as the progesterone receptor is a nuclear protein. The slides were washed, dehydrated and mounted (**3.2.8**) prior to analysis.

Steroidogenic cells were identified on serial sections using a specific polyclonal rabbit antibody to human 3β-HSD (**3.1.2**) as described previously (**7.3.5**). Briefly, fixed sections were prepared as described (**3.2.2**) and incubated with the primary antibody in a dilution of 1:1000. NGS was used to block non-specific binding, and specific antibody binding was detected using biotinylated goat anti-rabbit immunoglobulins (**3.2.6**) and visualised with an AB-HRP reaction which turned the substrate (DAB) into a stable brown end-product (**3.2.7**).

Immunohistochemistry for progesterone receptor isoforms was performed on frozen sections (**3.2.3**). Mouse monoclonal antibodies which recognised both 'A' and 'B' isoforms of the progesterone receptor, or solely the 'B' isoform of the receptor were used (**3.1.2**). The monoclonal antibody to the 'B' isoform of the receptor had no cross-reactivity with the 'A' isoform. Primary antibodies were used at a concentration of 1:50 and mouse IgG at the same concentration was used in place of the primary antibody as a negative control (**3.2.9**). NRS was used to block non-specific binding and specific antibody binding was detected using biotinylated rabbit anti-mouse immunoglobulins (**3.2.6**) and visualised with an AB-AP reaction which turned the substrate (NBT) into a stable blue end-product (**3.2.7**).

8.3.3 Analysis of Results

Progesterone receptors were quantified on immunostained sections by computer-aided nuclear densitometry (Koh *et al.*, 1995). Only sections from the same run performed in carefully controlled conditions were analysed together. Grey-scale densitometric analysis was performed after image capture using an image analysis program (NIH Image 1.55) using a stratified random sampling technique. The average grey scale reading on at least fifty nuclei in three fields of view per section was calculated, and the results monitored with reference to the running mean.

Background readings, taken from the cytoplasm of the same cells, were subtracted from the average grey scale reading to give a more objective measurement of staining intensity.

The validity of the technique was monitored by analysis of different immunohistochemistry runs and by repeating the measurements on the same sections at a later date. In the endometrium, nuclear staining glandular and stromal compartments varied little within the compartment: thus all identified nuclei were analysed. In the steroidogenic compartment of the corpus luteum, only a proportion of nuclei immunostained. In this case, the percentage of positive nuclei were counted, and densitometry was only performed on the positive nuclei. Results were analysed statistically by one-way ANOVA using a commercial software package (StatView 4.0). Where differences existed, with a 5% level of significance, pairwise comparisons were performed using the Bonferroni/Dunn method.

8.4 Results

8.4.1 Plasma Progesterone Concentrations

Mean plasma progesterone concentrations were 32.4 ±9.6 nmol/l in the early-luteal phase, 40.9 ±9.9 nmol/l in the mid-luteal phase, and 19.2 ±12.6 nmol/l in the late-luteal phase. Plasma progesterone concentrations in women receiving hCG rose from a mean of 38.2 ±3.4 nmol/l to a mean of 52.7 ±1.1 nmol/l at the time of surgery, confirming luteal 'rescue' in each case. The classification of the corpora lutea by serial urinary LH measurement agreed with the luteal phase dating of endometrial biopsies using the method of Li *et al.* (1988).

8.4.2 Endometrial Progesterone Receptor Immunostaining

Immunostaining for the progesterone receptor was exclusively nuclear in all sections investigated (Fig. 8.1*a*). No specific immunostaining was detected in serial negative control sections, where the primary antibody was replaced by non-specific immunoglobulins at the same concentration (Fig. 8.1*b*). Nuclear progesterone receptor immunostaining was seen in the glandular and stromal compartments of early-luteal phase endometrium (Fig. 8.1*a*). In contrast, progesterone receptor immunoreactivity was exclusively stromal in the mid- (Fig.

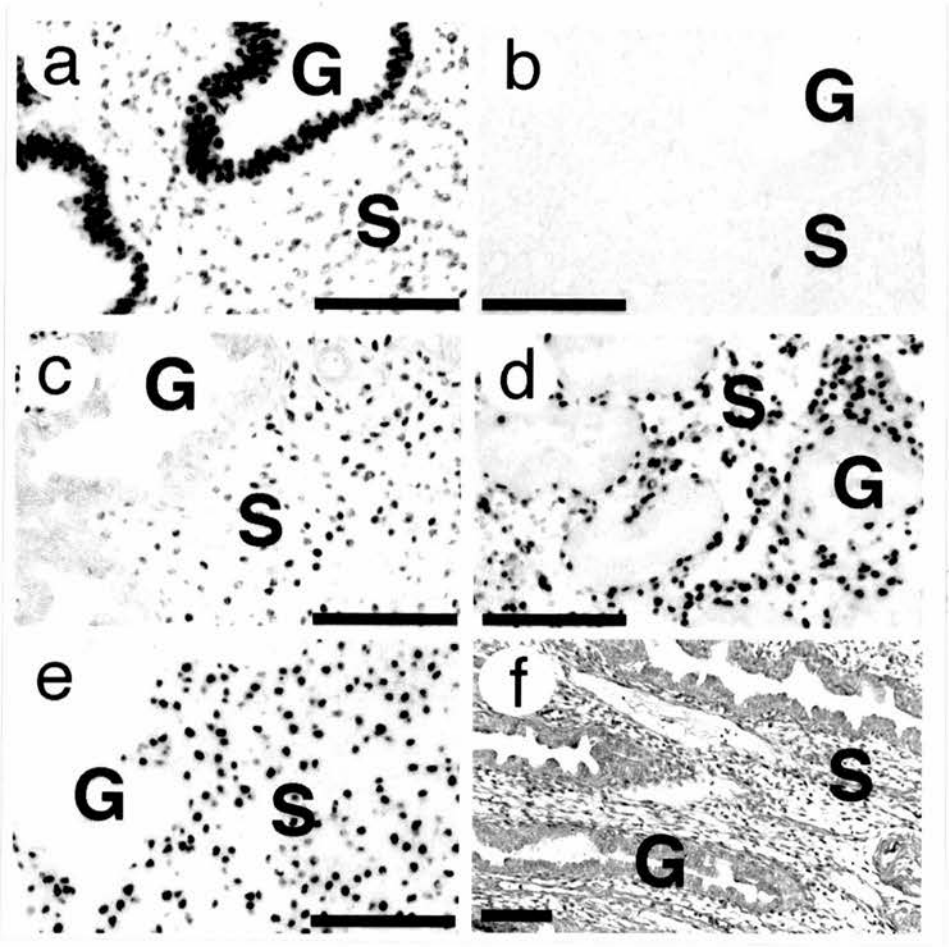


Figure 8.1

Progesterone receptor immunostaining in human endometrium

Immunohistochemistry for progesterone receptors in endometrium: **a)** early-luteal phase endometrium showing specific nuclear staining in the glandular (G) and stromal (S) compartments; **b)** negative control serial section of (a), where the primary antibody was replaced with non-specific immunoglobulins, showing no specific staining in the glands (G) or stroma (S); **c)** mid-luteal endometrium showing nuclear progesterone receptor immunostaining in the stroma (S) but not the glands (G); **d)** continued stromal (S) immunostaining and absence of glandular (G) staining in late-luteal endometrium; **e)** the same pattern of glandular (G) and stromal (S) immunostaining seen in the endometrium after luteal 'rescue' with exogenous hCG; **f)** haematoxylin and eosin stained section of 'rescued' endometrium showing some decidualisation of the stroma (S) and early Arias-Stella reaction of pregnancy in the glands (G). Scale bar = 100 μm.

8.1*c*) and late-luteal phase endometrium (Fig. 8.1*d*). After luteal 'rescue' with exogenous hCG, to maintain endometrial progesterone exposure in the late-luteal phase, progesterone receptor immunoreactivity remained exclusively stromal (Fig. 8.1*e*). After luteal 'rescue', the endometrium showed morphological evidence of decidualisation and the Arias-Stella reaction of pregnancy (Fig. 8.1*f*).

There was no change in the intensity of stromal nuclear progesterone receptor immunostaining throughout the luteal phase or after luteal 'rescue' with exogenous hCG (Fig. 8.2). However, there was a significant change in the intensity of glandular progesterone receptor immunostaining (Fig. 8.2). Glandular nuclear immunoreactivity was much lower ($p<0.005$) in the mid- and late-luteal endometrium than in the early-luteal endometrium. There was no difference in endometrial progesterone receptor immunostaining in the late-luteal phase, when progesterone exposure is declining, and the endometrium from simulated early pregnancy, when progesterone exposure is increasing (Fig. 8.2).

8.4.3 Luteal Progesterone Receptor Immunostaining

Nuclear progesterone receptor immunostaining could clearly be identified in luteinised periovulatory granulosa cells (Fig. 8.3*a*). In the mature corpus luteum progesterone receptors could be detected on a proportion of luteal cells (Fig. 8.3*b*). Comparison with serial sections immunostained for the steroidogenic enzyme 3β-HSD confirmed that progesterone-producing cells can express specific nuclear receptors to progesterone (Fig. 8.3*c*). Progesterone receptor immunoreactivity in some steroidogenic cells was seen in all sections studied. Progesterone receptor immunoreactivity was also present in cells of the surrounding stroma which was often more intense than the steroidogenic cell immunostaining (Fig. 8.3*d,e*).

When the intensity of steroidogenic cell progesterone receptor immunostaining was analysed, there were no significant differences throughout the luteal phase and after luteal 'rescue' with exogenous hCG (Fig. 8.4). In addition, there were no differences in the proportion of cells demonstrating immunodetectable progesterone receptors throughout the luteal phase or after luteal 'rescue' (Fig. 8.4). Immunohistochemistry for progesterone receptors resulted in a much lower intensity stain in the corpus luteum than in the endometrium under identical conditions (Fig. 8.5).

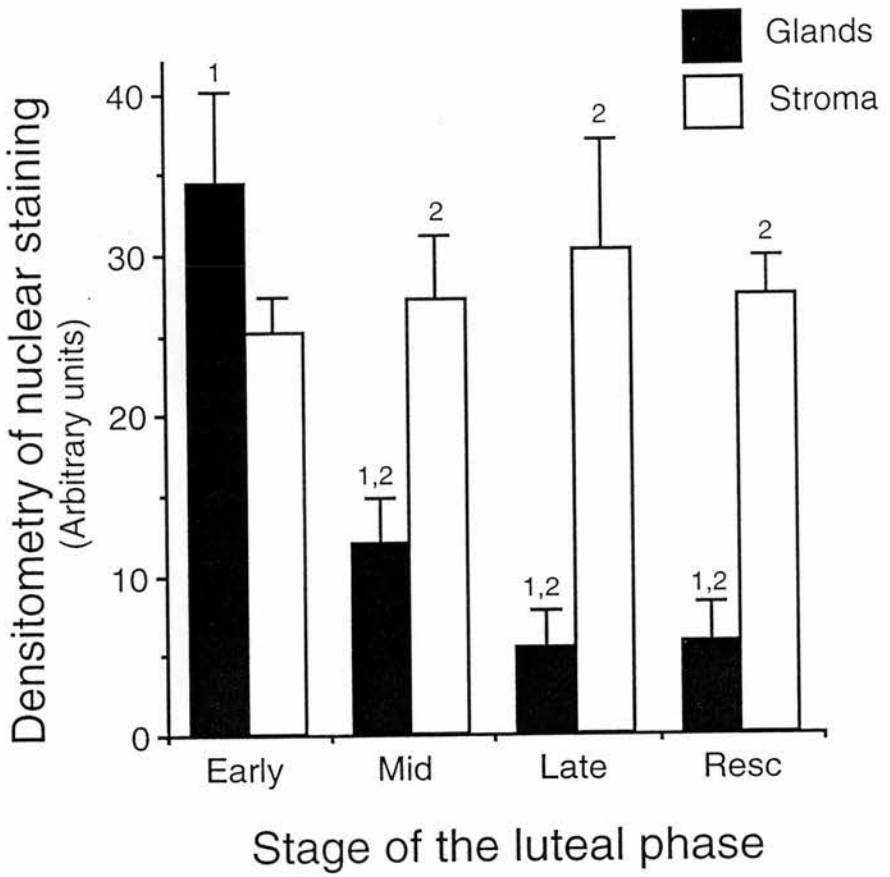


Figure 8.2

Quantification of endometrial progesterone receptor immunostaining

The intensity of progesterone receptor immunostaining as measured by nuclear densitometry in the glandular and stromal compartments of endometrium from the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late-luteal (LH+11 to LH+14) phases, and after luteal 'rescue' (hCGx5 to hCGx8) to mimic early pregnancy (n=6 per group; values are mean ±S.E.M.). Immunostaining in the glandular nuclei was highest in the early-luteal phase ($p<0.005$; ANOVA). There was no difference in the staining of glandular and stromal nuclei in the early-luteal phase but in the mid- and late-luteal phases, and after luteal 'rescue' with exogenous hCG stromal immunoreactivity was greater than glandular ($p<0.001$; ANOVA). There was no difference in stroma cell nuclear progesterone receptor immunostaining across the luteal phase or after luteal 'rescue'.

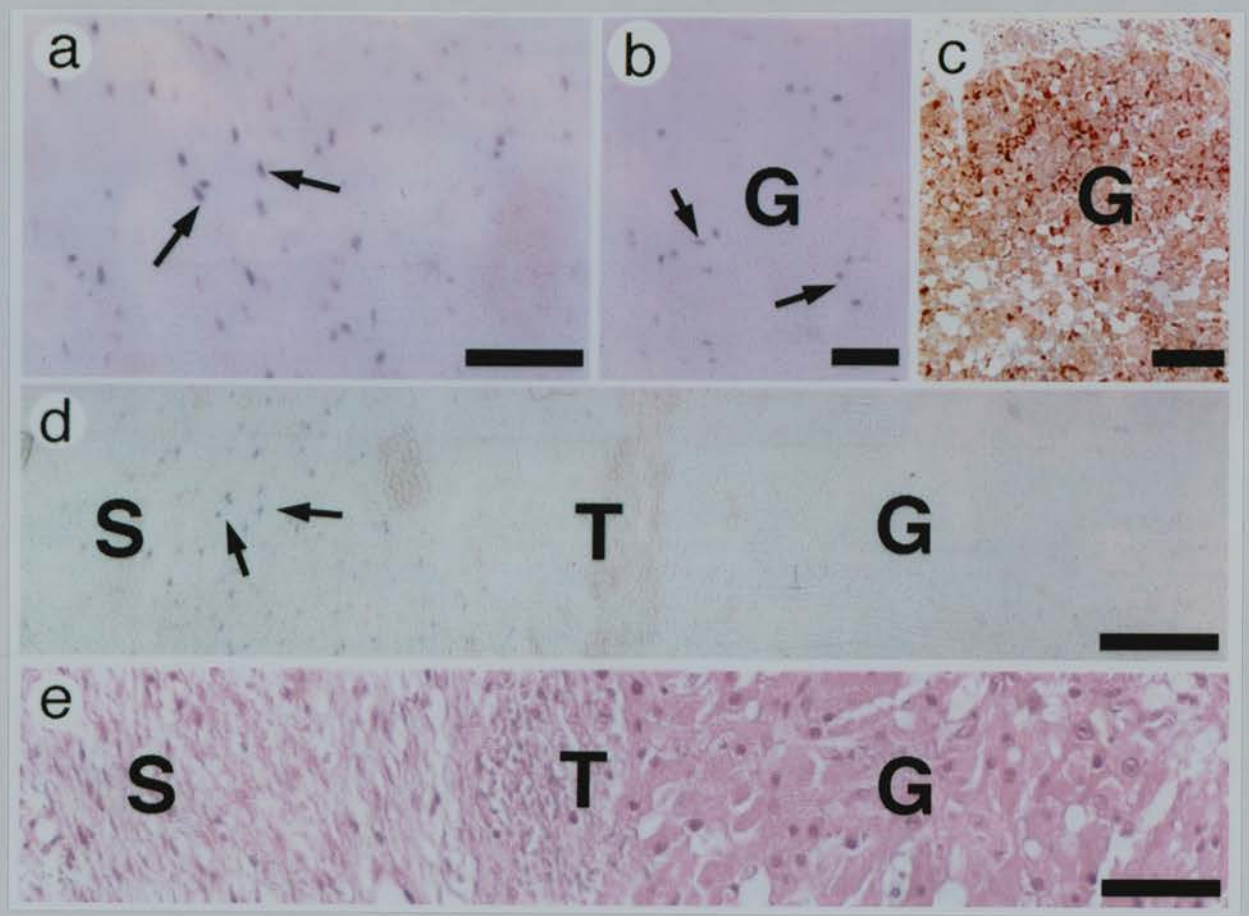


Figure 8.3

Progesterone receptor immunostaining in human corpora lutea

Immunohistochemistry for progesterone receptors in corpora lutea: **a)** luteinised granulosa cells, collected just prior to follicular rupture, showing specific nuclear immunostaining for progesterone receptors (arrows); **b)** mid-luteal corpus luteum showing scattered nuclear progesterone receptor staining (arrows) within the granulosa-lutein cell layer (G); **c)** section from the same corpus luteum as (b) immunostained for the steroidogenic enzyme 3β-HSD, showing these cells, in the granulosa-lutein layer (G), to be steroidogenic in nature; **d)** late-luteal corpus luteum showing specific nuclear progesterone receptor immunostaining in the non-steroidogenic stromal tissue (S) surrounding the steroidogenic granulosa-lutein cells (G) and theca-lutein (T) cells, which display less marked nuclear progesterone receptor immunostaining; **e)** section from the same corpus luteum as (d) stained with haematoxylin and eosin to clarify the architecture of the steroidogenic granulosa-lutein cells (G), theca-lutein cells (T) and the surrounding stromal tissue (S). Scale bar = 100 μm.

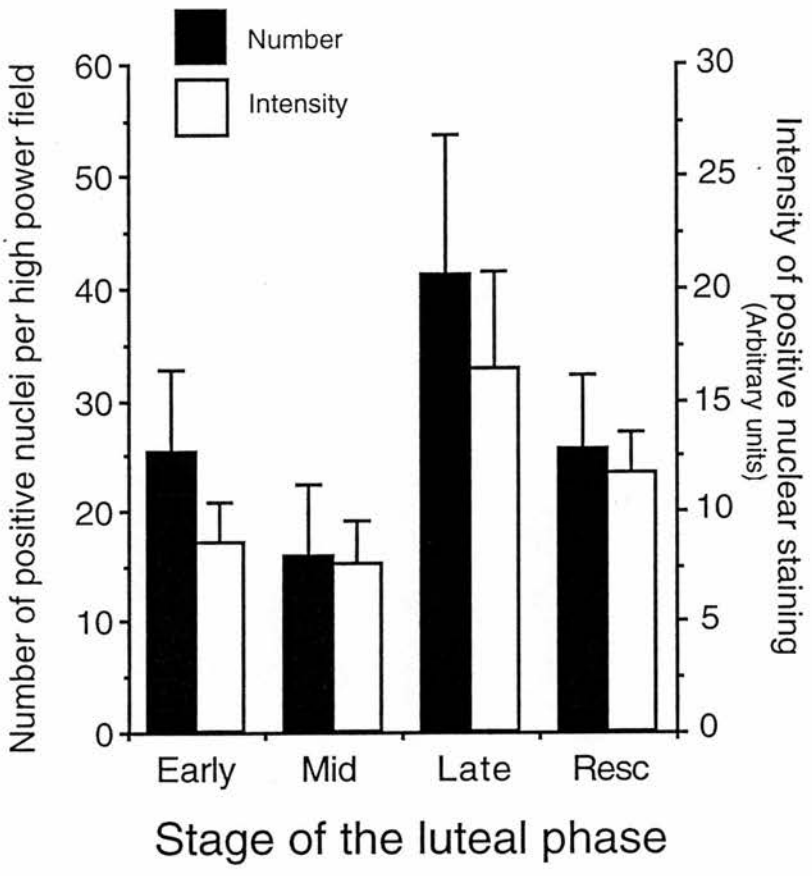


Figure 8.4

Quantification of luteal progesterone receptor immunostaining

The mean number of nuclei demonstrating specific progesterone receptor immunoreactivity per high power field, in the steroidogenic cells of human corpora lutea, and their mean intensity of immunostaining measured by nuclear densitometry are shown (n=6 per group; values are mean ±S.E.M.). There were no differences in the number or intensity of stained nuclei in corpora lutea from the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late-luteal (LH+11 to LH+14) phases or after luteal 'rescue' with exogenous hCG (hCGx5 to hCGx8) to simulate the hormonal changes of early pregnancy (ANOVA).

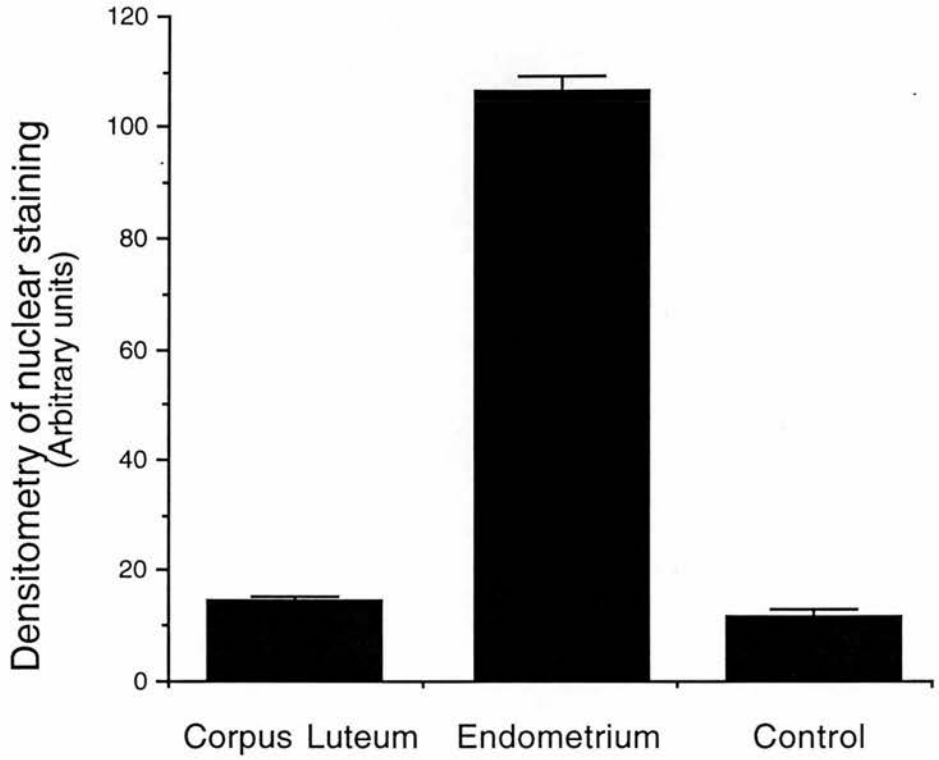


Figure 8.5

Comparison of immunostaining intensity in the endometrium and corpus luteum

The intensity of progesterone receptor immunostaining as measured by nuclear densitometry in endometrial stroma (Endometrium, n=24) and granulosa-lutein cells of corpora lutea (Corpus Luteum, n=24). These are compared to nuclear densitometry of granulosa-lutein cells of the control sections where mouse immunoglobulins were used in place of the primary antibody (Control, n=24). This shows the nuclear progesterone receptor immunoreactivity to be much greater in endometrium than corpora lutea under these conditions (values are means ±S.E.M.).

8.4.4 Identification of the 'B' Isoform of the Progesterone Receptor

Immunohistochemistry for the 'B' isoform of the progesterone receptor was performed on frozen sections of corpora lutea. Specific nuclear immunostaining for the 'B' progesterone receptor could be detected in luteal steroidogenic cells (Fig. 8.6*a*). Comparison of serial sections immunostained with the same concentration of a monoclonal antibody, prepared under the same conditions, which recognised both the 'A' and 'B' forms of the receptor showed a similar pattern and intensity of staining (Fig. 8.6*b*). This pattern of immunostaining was repeated in all sections throughout the luteal phase and after luteal 'rescue' with exogenous hCG (Fig. 8.6*c,d*). Immunoreactive nuclear progesterone receptors appeared to localise to a greater proportion of steroidogenic luteal cells when frozen sections were analysed with these antibodies. However, as tissue definition was much poorer, quantitative analysis was not performed.

8.5 Discussion

This study reports the expression and localisation of the progesterone receptor in human endometrium and corpora lutea throughout the luteal phase and after luteal 'rescue' with exogenous hCG. Expression of the progesterone receptor in human endometrium has been well documented previously (Press *et al.*, 1988; Lessey *et al.*, 1988). The localisation of specific progesterone receptor immunoreactivity in our study agrees with these reports. These data suggest that the progesterone receptor in stromal cells of the endometrium is regulated differently from the progesterone receptor in endometrial glands. Whereas the glandular receptor appears to be down-regulated by progesterone, this is not true of the stromal receptor during the normal secretory phase. Progesterone receptor immunoreactivity has been reported in the stromal cells of decidua of early pregnancy (Perrot-Applanat *et al.*, 1994). Using a luteal 'rescue' model, which allows prolonged exposure to progesterone in the absence products from the conceptus, we have confirmed the continued expression of the stromal cell progesterone receptor in the presence of increasing progesterone concentrations.

There is increasing evidence for the expression of progesterone receptors in the steroidogenic cells of the primate corpus luteum. Press and Greene (1988) first used immunohistochemistry to detect progesterone receptors in the human corpus

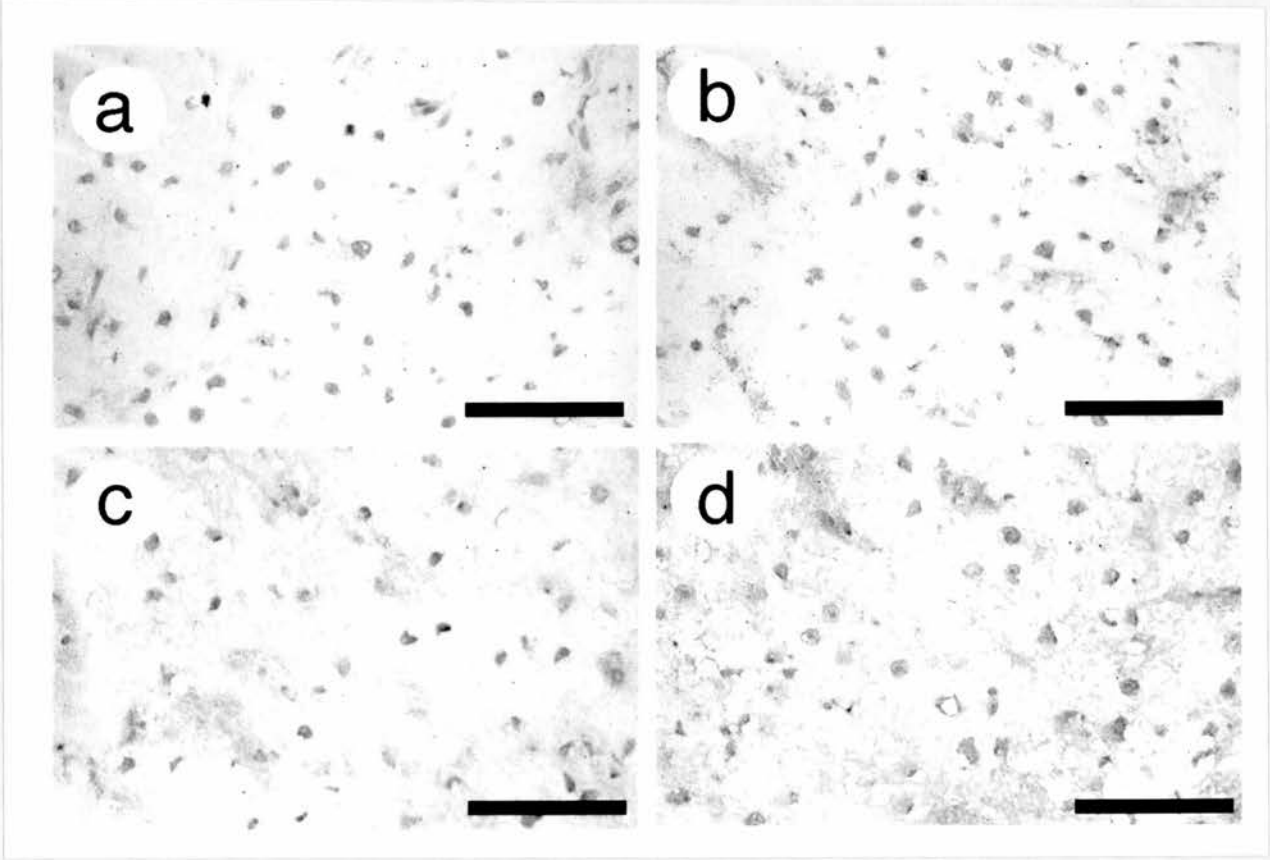


Figure 8.6

Immunolocalisation of the 'B' isoform of the progesterone receptor in corpora lutea

Immunohistochemistry for progesterone receptor isoforms in the granulosa-lutein cells of human corpora lutea: **a)** early-luteal corpus luteum showing specific nuclear reactivity after immunostaining with an antibody which solely recognises the 'B' isoform of the progesterone receptor; **b)** serial section of (a) showing the same pattern of nuclear immunoreactivity after staining with an antibody which recognises both the 'A' and the 'B' isoforms of the progesterone receptor; **c)** section of a corpus luteum after luteal 'rescue' with exogenous hCG, immunostained for the 'B' isoform of the progesterone receptor showing continued nuclear immunoreactivity; **d)** serial section of (c) after staining for both the 'A' and 'B' isoforms of the progesterone receptor showing the same pattern of nuclear immunoreactivity. Scale bar = 100 μm.

luteum. Since then, several studies have systematically immunolocalised progesterone receptors to the steroidogenic cells of the corpus luteum of the human (Iwai *et al.*, 1990; Horie *et al.*, 1992; Suzuki *et al.*, 1994), rhesus monkey (Hild-Petito *et al.*, 1988) and baboon (Hild-Petito and Fazleabas, 1997). Other techniques have been used to confirm luteal progesterone receptor expression in the primate, including radioligand binding assays (Slayden *et al.*, 1994), western blotting (Duffy *et al.*, 1997), RT-PCR (Chandrasekher *et al.*, 1994), northern blotting (Hild-Petito and Fazleabas, 1997) and RNAse protection assay (Duffy and Stouffer, 1995). We have again immunolocalised progesterone receptors to the primate corpus luteum. This study continues the line of evidence that the progesterone-producing cells of the corpus luteum have the potential to respond to that progesterone in an autocrine fashion.

As well as confirming the expression of progesterone receptors in the corpus luteum, this study gives some additional pieces of information. Firstly, we found that the progesterone receptor was much harder to immunolocalise in the corpus luteum than in the endometrium, a classical progesterone-responsive tissue. In the one study where progesterone receptors were immunolocalised in a variety of tissues, the immunostaining of the ovary and endometrium were not compared or contrasted (Press and Greene, 1988). This suggests that either progesterone receptors are expressed in low levels by the corpus luteum or that tissue fixation differences affect the tissues' immunostaining properties. Whereas, tissue antigen accessibility may well be involved, there is indirect evidence that this may not be the case. We found no differences in immunostaining properties in the same endometrial and luteal tissues when bcl-2 localisation was investigated (Rodger *et al.*, 1995; Koh *et al.*, 1995). In addition, progesterone receptor immunostaining was easier to detect in the surrounding stroma than the steroidogenic cells of the same corpus luteum. Immunostaining of luteal stromal cells has previously been well documented (Press and Greene, 1988; Suzuki *et al.*, 1994). Western blotting (Duffy *et al.*, 1997) and RNAse protection assays (Duffy and Stouffer, 1995) for progesterone receptors recently demonstrated much lower expression than in an equivalent amount of endometrium. It is probable that steroidogenic cells of the corpus luteum express much lower levels of progesterone receptors than classical progesterone target tissues.

We found that the 'rescued' corpus luteum continued to express progesterone receptors. Previous studies of luteal progesterone receptors in the human (Iwai *et*

al., 1990, Horie *et al.*, 1992; Suzuki *et al.*, 1994) and monkey (Hild-Petito *et al.*, 1988) looked at corpora lutea from normal ovarian cycles. Prior to ovulation, LH stimulates the expression of progesterone receptors in granulosa cells (Chandrasekher *et al.*, 1991). *In vitro*, it has been suggested that hCG also increases the expression of progesterone receptor mRNA in luteinised monkey granulosa cells (Duffy *et al.*, 1996). We used exogenous hCG to simulate the luteal events of early pregnancy in the human. The increased progesterone receptor expression seen after the LH surge does not follow exposure to hCG in the corpus luteum. The luteal progesterone receptor is not up-regulated or down-regulated by prolonged exposure to progesterone during luteal 'rescue'. A recent study has confirmed the continued expression of progesterone receptors in baboon corpora lutea collected during early pregnancy (Hild-Petito and Fazleabas, 1997). The corpus luteum of early pregnancy, therefore, maintains the potential to respond to the progesterone it continues to produce.

We looked at the expression of progesterone receptor isoforms in the corpus luteum. There is evidence that different isoforms of the human progesterone receptor have different functional properties (Graham and Clarke, 1997). It is thought that the 'B' isoform of the progesterone receptor mediates the stimulatory effects of progesterone in classical target tissues (Vegeto *et al.*, 1993). The 'A' isoform can inhibit the ability of the 'B' isoform of the progesterone receptor to stimulate transcription (Vegeto *et al.*, 1993; Tung *et al.*, 1993). Although it is not possible to specifically immunolocalise the 'A' isoform of the receptor, we found it likely that the main isoform in the corpus luteum was the 'B' progesterone receptor. Recently, using western blotting Duffy *et al.* (1997) confirmed that the 'B' progesterone receptor was the main isoform in the monkey corpus luteum, particularly in the late-luteal phase. This suggests that it is the receptor isoform associated with classical progesterone action that is primarily present in the corpus luteum.

There has been some controversy about changes in progesterone receptor expression in the corpus luteum throughout the luteal phase. Some studies have suggested that progesterone receptor expression is reduced in the late-luteal phase (Hild-Petito *et al.*, 1988; Iwai *et al.*, 1990; Suzuki *et al.*, 1994). We did not find any change in the level of progesterone receptor immunoreactivity throughout the luteal phase. It is uncertain why this discrepancy is present. It may relate to the classification of corpora lutea, which has previously been morphological in basis.

We have used carefully controlled urinary tracking to accurately date each corpus luteum on the basis of the urinary LH surge. Indeed, it has been reported that progesterone receptor mRNA is present in the corpus luteum throughout the luteal phase (Chandrasekher *et al.*, 1994; Duffy and Stouffer, 1995; Hild-Petito and Fazleabas, 1997), as is the protein as detected by western blotting (Duffy *et al.*, 1997). Although not fully resolved, it appears that progesterone receptors can be detected in primate corpora lutea throughout their functional lifespan.

It is unclear whether all, or a proportion of the steroidogenic cells of the corpus luteum express progesterone receptors. In this study, only a proportion of luteal cells had immunodetectable progesterone receptors. This has also been seen in other immunohistochemical studies (Press and Greene, 1988; Duffy *et al.*, 1994; Hild-Petito and Fazleabas, 1997). Other studies have demonstrated specific immunostaining in a greater proportion of steroidogenic cells (Hild-Petito *et al.*, 1988; Suzuki *et al.*, 1994; Slayden *et al.*, 1994). We found a greater percentage of luteal cells expressed progesterone receptors, when we used frozen sections and different primary antibodies. This difference is likely to be due to different sensitivities of the various immunohistochemical techniques. It is therefore likely that progesterone receptors are expressed by the majority of the steroidogenic cells in the corpus luteum.

The role of the luteal progesterone receptor is still not clear. Rat corpora lutea do not express progesterone receptors (Parke-Sarge *et al.*, 1995). The physiology of luteolysis and maternal recognition of pregnancy is markedly different in rats (Niswender and Nett, 1994). As progesterone receptors are present in the primate, this may mean that they are required for a specific functional role. It is not fully established whether other non-primate species, with alternate mechanisms to control luteal function, express progesterone receptors in their corpora lutea. There has however been a preliminary report that the ovine corpus luteum expresses progesterone receptors (Smith *et al.*, 1995b). Although the reasons are not clear, this species difference in luteal progesterone receptor expression may give us clues as to its function.

Several approaches have been used to determine whether progesterone has a functional role in the primate corpus luteum. The effect of administration of the antigestogen RU486 on luteal function was studied initially. It has been shown that RU486 can inhibit normal luteal function in women (Schaison *et al.*, 1985; Garzo *et al.*, 1988). However, this is likely to be directly related to its effects of

pituitary gonadotrophins (Schaison *et al.*, 1985; Garzo *et al.*, 1988; Batista *et al.*, 1994). When the corpus luteum is supported by exogenous hCG in the presence of RU486, normal luteal progesterone production continues, in spite of uterine bleeding (Croxatto *et al.*, 1989). In addition, *in vivo*, RU486 administration had no effect on progesterone production from cultured luteinised granulosa cells, which expressed immunodetectable progesterone receptors (Greenberg *et al.*, 1990). It seems that either progesterone has little effect on luteal function directly or other techniques are required to dissect its role.

One novel approach to study the local effects of progesterone is to administer trilostane, a 3β-HSD inhibitor. Duffy *et al.* (1994) administered trilostane to rhesus monkeys during the luteal phase. They found no effect on serum gonadotrophin levels but low progesterone levels, which did not recover after trilostane withdrawal. They concluded progesterone may be required for the maintenance of the functional and structural integrity of the primate corpus luteum (Duffy *et al.*, 1994). However, in this model oestrogen production was unexpectedly maintained, as was the production of other luteal products, such as relaxin (Duffy *et al.*, 1995; Duffy *et al.*, 1996). Although there is some evidence from this model that progesterone may modulate the expression of its receptor (Duffy and Stouffer, 1995), we did not see a change in progesterone receptors across the luteal phase in the presence of different progesterone concentrations. It is possible that progesterone has direct effects on LH receptor expression. *In vitro* progesterone treatment of bovine luteal cells resulted in increased LH receptor expression (Jones *et al.*, 1992). The LH receptor, however, is expressed in the human corpus luteum throughout the luteal phase in the presence of differing progesterone concentrations (Duncan *et al.*, 1996a). The concept of a local effect of progesterone on luteal function is attractive (Rothchild, 1981; Rothchild, 1996), but at present there are no direct functional data to fully explain the role of the luteal progesterone receptor.

In conclusion, we have shown that the progesterone receptor can be immunolocalised to the human corpus luteum throughout the luteal phase and after luteal 'rescue' with exogenous hCG. It is likely that the level of expression of these receptors is less than that in the classical progesterone-responsive tissues, but the isoform is that associated with a functional effect. The functional role of the progesterone receptor of the corpus luteum is not known. It is possible that progesterone has a role in regulating its own synthesis. Our findings, that the low

levels of progesterone receptors do not vary in the different functional stages of the lifespan of the corpus luteum, despite varying progesterone concentrations, do not support the hypothesis that progesterone receptors have a major functional role in regulating luteal progesterone production.

Chapter 9

Experimental Section II: Structural Effects

9.1 General Introduction

This second section explores the structural remodelling of the corpus luteum at different stages of its lifespan. The mid-luteal corpus luteum is an extraordinarily active endocrine gland. It synthesises and secretes large amounts of steroids, non-steroid hormones and growth factors (Behrman *et al.*, 1993). It is, weight for weight the most active endocrine gland in the body (Rothchild, 1981) with a blood supply per unit mass many times that of the kidney (Ford *et al.*, 1992). However, the corpus luteum is a transient structure, with a life-span, under normal conditions, of little more than 14 days (Lenton and Woodward, 1988). At the time of menstruation, it is difficult to find the residual corpus luteum on the human ovary.

During its short lifespan the corpus luteum changes greatly from a structural point of view. Before ovulation, the dominant follicle is largely a fluid-filled cystic structure on the ovary measuring somewhere between 15 to 22 mm in diameter (Adashi, 1994). The granulosa cells lining the follicle are avascular in nature and obtain their nutrients by diffusion from blood vessels in the adjacent theca cell layer (Gougeon, 1996). This limits the thickness of the granulosa cell layer to five or six cells (Zeleznik and Fairchild Benyo, 1994). After ovulation, the follicular remnants are transformed into the highly vascular corpus luteum. The granulosa cell layer undergoes hypertrophy and is invaded by endothelial cells, supporting fibroblasts and connective tissue (Behrman *et al.*, 1993; Zeleznik and Fairchild Benyo, 1994). This stage of luteal development is associated with intense angiogenesis (Reynolds *et al.*, 1992) and endothelial cell proliferation (Rodger *et al.*, 1997). In the mid-luteal phase, the corpus luteum is a solid ball of tissue on the ovarian surface, around 15 mm in diameter. During luteolysis, this changes into a small relatively avascular fibrous remnant in a matter of days (Corner, 1956). The cell and tissue debris generated during the luteolytic process are thought to be removed by tissue macrophages (Paavola, 1979; Norman and Brännström, 1994). The numbers of macrophages are thought to increase in the corpus luteum during luteolysis (Best *et al.*, 1996), but the effect of luteal 'rescue' is not known.

As well as intense tissue remodelling around the time of ovulation, when the corpus luteum is formed, there is marked tissue remodelling during luteolysis (Luck and Zhao, 1995). It is becoming increasingly clear that the remodelling associated with luteolysis involves the loss of cells from the corpus luteum (Spencer *et al.*, 1996). It is now thought that this cell loss involves the death of both steroidogenic and endothelial cells by apoptosis (Juengel *et al.*, 1993; Fraser *et al.*, 1995b; Shikone *et al.*, 1996; Young *et al.*, 1997). This cell death is not seen during early pregnancy (Shikone *et al.*, 1996), and there is preliminary evidence that rates of luteal cell death are inhibited by hCG (Dharmarajan *et al.*, 1994). Much less is known about the remodelling of the ECM throughout the luteal phase and the effect of hCG during luteal 'rescue' on ECM synthesis and breakdown.

The connective tissue of a gland is made up primarily of ECM. The ECM consists of structural proteins such as collagen, proteoglycans, laminin, elastin and fibronectin which hold the cellular components of the gland together. It also supports the blood and lymph vessels, transient cell populations and forms a repository for growth factors (Hulboy *et al.*, 1997). Any change in the structure of a tissue involves synthesis and breakdown of the ECM. Little is known about tissue remodelling in the corpus luteum. Proteolytic breakdown of the ECM requires the action of powerful enzymes. The enzymes capable of denaturing fibrillar collagens are a group of zinc-dependent enzymes known as the MMPs (Matrisian, 1990; Woessner, 1991; Birkendal-Hansen, 1995). Indeed, studies of tissue remodelling in tumours during tissue invasion (Naylor *et al.*, 1994), the endometrium at menstruation (Salamonsen and Woolley, 1996; Hulboy *et al.*, 1997), and the follicle wall at ovulation (Reich, 1991; Curry *et al.*, 1992), have all implicated MMP action. It is therefore likely that MMPs are involved in the remodelling associated with luteolysis.

The MMPs are a group of at least 17 structurally related enzymes (Hulboy *et al.*, 1997) which are tightly controlled under physiological conditions (Birkedal-Hansen, 1995). Although most are secreted into the ECM (Woessner, 1991), the most recently described MMPs are transmembrane enzymes expressed on cellular membranes (Hulboy *et al.*, 1997). Although, there are several different enzymes, only a few have been extensively studied. These are the enzymes which can degrade the major structural proteins, and include MMP-1, MMP-2 and MMP-9 (Hulboy *et al.*, 1997). There is some preliminary evidence that these enzymes can be detected in infraprimate corpora lutea (Tsang *et al.*, 1995; Nothnick *et al.*,

1996), and luteinised human granulosa cell cultures (Puistola *et al.*, 1995; Aston *et al.*, 1996a). Indeed, one study suggested that their expression may be increased during prolactin-induced luteolysis in the rat (Endo *et al.*, 1993a). However, at present, there are only limited data about MMP expression and control in the corpus luteum, and no data about their expression in the primate, or the effect of maternal recognition of pregnancy. If their expression is important during luteolysis, it is important to determine if they are inhibited during luteal 'rescue'.

More is known about the expression of TIMPs in the corpus luteum. At present there are four members of the TIMP family. TIMP-1 and TIMP-2 are secreted proteins which are widely expressed (Hulboy *et al.*, 1997). TIMP-3 is found in association with the basement membrane (Uria *et al.*, 1994), and TIMP-4 has only recently been reported, and therefore much less well defined (Hulboy *et al.*, 1997). TIMPs bind to and inhibit MMP enzymes with a one-to-one stoichiometry (Woessner, 1991). TIMP-1 is thought to be a major secretory product of the corpus luteum. Although nothing is known of its expression in primate corpora lutea, it is produced in large amounts by the corpus luteum of pigs (Smith MF *et al.*, 1994), sheep (Smith GW *et al.*, 1994), cows (Juengel *et al.*, 1994) and rats (Nothnick *et al.*, 1995). It can also be detected in cultures of luteinised human granulosa cells (Rapp *et al.*, 1990; Morgan *et al.*, 1994). In addition, it is now clear that TIMP-2 can also be detected in infraprimate corpora lutea (Nothnick *et al.*, 1995; Smith *et al.*, 1995a; Smith *et al.*, 1996a). It is therefore likely that TIMPs have significant roles in the control of luteal structure or function.

These roles are not entirely clear. It is likely that TIMPs inhibit MMP activity in the corpus luteum (Salamonsen, 1996). However, there is no clear evidence for a reduction in TIMP expression during luteolysis (Juengel *et al.*, 1994). There may be other reasons why TIMPs are highly expressed by corpora lutea. TIMP-1 has been shown to have growth factor-like activity (Hayakawa *et al.*, 1992), share a degree of structural homology with bovine StAR (Hartung *et al.*, 1995), and act as a stimulator of steroidogenesis in the testicular cell cultures (Boujrad *et al.*, 1995). It is possible that these are also important roles for TIMP-1 in the corpus luteum. In addition, although it is clear that there are large amounts of TIMP-1 in the corpus luteum, it is not immediately clear how MMPs can function in the presence these large amounts of specific inhibitor.

There are therefore some major questions still to be answered about remodelling in the corpus luteum. Nothing is known about MMP and TIMP expression in the

primate corpus luteum. Nothing is known about the effect of luteal 'rescue' during maternal recognition of pregnancy on TIMP and MMP expression. Nothing is known about the localisation of MMPs and TIMPs in relation to each other in the corpus luteum and which cell types are responsible for their production. In addition, the role of TIMP-1 expression in the corpus luteum is unclear. Is it principally to inhibit MMPs or are there other roles? For example, does TIMP-1 act as a steroidogenic factor? Clearly, the investigation of structural remodelling in the corpus luteum is important. At present little is known, and this area has great potential for study.

9.2 Scope of the Section

This section aims to investigate the factors involved in tissue remodelling in the corpus luteum during luteolysis and during luteal 'rescue'. The following four chapters aim to systematically address the factors involved in structural remodelling of the ECM of the primate corpus luteum, throughout its functional lifespan, with particular emphasis on luteal 'rescue'. Like the previous experimental section, each chapter is written in the form of a scientific paper. The conclusions of this section will be drawn together at the end of this thesis. As mentioned previously, the results of the chapters in both the experimental sections of this thesis will be drawn together in **Chapter 14** in an attempt to describe a unifying paradigm.

The first area to be addressed in this section is the expression of TIMP-1 in the primate corpus luteum. TIMP-1 specifically inhibits the MMP enzymes involved in tissue remodelling processes throughout the body. **Chapter 10** addresses whether TIMP-1 can be localised to human corpora lutea and whether the inhibition of structural luteolysis during luteal 'rescue' is associated with an alteration in the expression of TIMP-1. The potential alteration of TIMP-1 expression during luteolysis is further investigated in **Chapter 11**. The ontogeny of TIMP-1 expression in the corpus luteum is also addressed in **Chapter 11** by studying its expression in the steroidogenic cells of the follicle during their growth and development. A potential steroidogenic role for TIMP-1 is addressed in **Chapter 11** by studying its expression in a variety of steroidogenic and non-steroidogenic tissues.

The second area of remodelling to be investigated is the expression of MMP enzymes in the human corpus luteum. **Chapter 12** assesses the expression and activity of the major MMPs in the corpus luteum, throughout the luteal phase and after luteal 'rescue' with hCG to prevent structural luteolysis. **Chapter 12** also addresses the expression of the other members of the TIMP family in relation to the MMPs. The mechanism whereby MMPs can function in an environment containing TIMPs are also addressed in **Chapter 12** by studying the tissue localisation of these enzymes and their specific inhibitors.

The third factor in the remodelling process to be investigated is the involvement of immune cells. **Chapter 13** investigates the main immune cell in the corpus luteum, the macrophage. The numbers, and specific tissue localisations, of macrophages in the corpus luteum are assessed throughout the functional luteal phase. **Chapter 13** also reports the effect of hCG, during luteal 'rescue', on the number of macrophages in the corpus luteum. The mechanism of the effect of hCG on macrophage numbers is therefore investigated, by comparing the localisation of macrophages with that of the LH/hCG receptor.

Chapter 10

Expression of TIMP-1 in the human corpus luteum after luteal 'rescue'

10.1 Abstract

TIMP-1 is a specific inhibitor of a group of proteolytic enzymes known as MMPs. These enzymes have been widely implicated in the process of tissue remodelling. Extensive remodelling occurs in the corpus luteum during luteolysis unless hCG is produced by the early conceptus. This study aimed to investigate the expression and localisation of TIMP-1 in human corpora lutea during the luteal phase of the cycle and after luteal 'rescue' with exogenous hCG, to mimic the changes of early pregnancy. Human corpora lutea from the early- (n=4), mid- (n=4) and late- (n=4) luteal phases, and after luteal 'rescue' by hCG (n=4), were obtained at the time of hysterectomy. Expression of TIMP-1 was investigated in these tissues by western blotting, immunohistochemistry, northern blotting and *in situ* hybridisation. Luteal cells of thecal origin were distinguished from those of granulosal origin by immunostaining for 17α-hydroxylase. A 30 kilodalton protein consistent with TIMP-1 was detected in human corpora lutea. This protein was localised to the granulosa-lutein cells in all tissues examined. TIMP-1 mRNA was found in large quantities in all glands examined and this again localised to the granulosa-lutein cells. The expression and localisation of TIMP-1 did not change throughout the luteal phase and was not altered by luteal 'rescue'. The function of this uniform expression of TIMP-1 in the corpus luteum is not clear but these data suggest that the inhibition of structural luteolysis during maternal recognition of pregnancy is not mediated by regulation of TIMP-1 expression.

10.2 Introduction

The human corpus luteum will undergo functional and structural luteolysis unless hCG is produced by the implanting blastocyst (Behrman *et al.*, 1993). Although this process is fundamental to ovarian function, the molecular mechanisms of luteolysis and luteal 'rescue' in the human are still uncertain. During structural luteolysis the gland rapidly changes from the most active endocrine gland in the body, with a greater blood flow per unit mass than the kidney (Ford *et al.*, 1982), to a small fibrous remnant.

Such a process is likely to involve extensive remodelling of the ECM that is controlled by MMPs, a group of zinc-dependant proteolytic enzymes. Alterations in MMP activity have been implicated in the spread of neoplastic tissues (Naylor *et al.*, 1994) as well as in remodelling of reproductive tissues such as the endometrium at menstruation (Hampton and Salamonsen, 1994) and the follicle wall at ovulation (Reich *et al.*, 1985). The activity of MMPs is rigorously controlled at several levels, including synthesis as a pro-enzyme, requiring activation, and the production of specific inhibitors: TIMP-1, TIMP-2 and TIMP-3. TIMP-1 is of particular interest as it has recently been demonstrated that it is the major secretory product of the ovine corpus luteum (Smith *et al.*, 1993) and that its expression is transiently increased during $PGF_{2\alpha}$ induced luteolysis (Juengel *et al.*, 1994). Other studies have reported that TIMP-1 expression increases markedly in preovulatory granulosa cells around the time of the gonadotrophin surge (Smith GW *et al.*, 1994).

This study aimed to investigate the role of TIMP-1 in the human corpus luteum by studying the expression in the corpus luteum through both the normal luteal phase and during early pregnancy simulated by the administration of hCG in logarithmically increasing doses.

10.3 Specific Materials and Methods

10.3.1 Tissues Studied

Corpora lutea were enucleated at the time of hysterectomy in 16 women undergoing surgery for benign conditions (**2.2.3**). Four corpora lutea were classified as early-luteal, four as mid-luteal and four as late-luteal (**2.2.4**). Four

corpora lutea were obtained after exogenous hCG administration to 'rescue' the corpus luteum (**2.2.2**). Two pieces of each corpus luteum were stored at -70 °C for subsequent RNA and protein extraction (**2.2.3**). One piece was fixed in 4% paraformaldehyde and embedded in paraffin wax (**2.2.3**) for subsequent immunohistochemistry (**3.2**) and another piece was frozen in embedding medium for the preparation of frozen sections (**2.2.3**). In each case an endometrial biopsy was also fixed in paraformaldehyde (**2.2.3**) and plasma was taken before surgery for estimation of progesterone concentration (**2.2.2**).

10.3.2 Immunohistochemistry

Paraffin wax sections (5 μm) on poly-L-lysine-coated slides (**3.2.1**) were de-waxed and rehydrated (**3.2.2**). As preliminary experiments indicated that microwave antigen retrieval was necessary for the detection of TIMP-1, all sections were therefore microwaved (**3.2.4**). Non-specific binding was blocked by NRS containing 5% (w/v) BSA (**3.2.6**). Sections were incubated overnight at 4 °C with a 1:20 dilution of monoclonal mouse anti-TIMP-1 antibody (**3.1.2**) in TBS (**3.2.6**). This antibody has less than 0.01% cross-reactivity with TIMP-2 protein. Antibody binding was indicated using AB-AP (**3.2.7**) with substrate to give a red end product (**3.2.7**). As negative controls, serial sections were incubated with mouse IgG in place of the primary antibody at the same concentration (5 μg/ml) (**3.2.9**) and in further control sections the primary antibody was omitted (**3.2.9**). The intensity of staining was graded by an observer blinded to the tissue identity. Staining was classified as, - if absent, + if weakly present, ++ if moderate and +++ if strong.

Serial sections were also stained for 17α-hydroxylase using a polyclonal rabbit antibody (**3.1.2**) at 1:750 dilution in TBS. In this case, after microwave antigen retrieval (**3.2.4**), NGS was used to block non-specific binding and specific binding was detected using biotinylated goat anti-rabbit immunoglobulins (**3.2.6**).

10.3.3 Western Blotting

Two hundred micrograms of protein (**3.3.1**) was denatured, separated by electrophoresis on an 11% polyacrylamide gel (**3.3.1**) and electrophoretically transferred onto nitrocellulose membrane (**3.3.1**). Primary antibody (**3.1.2**) was used in a concentration of 2 μg/ml in TBST (**3.3.2**). Specific antibody binding was visualised with biotinylated goat anti-rabbit immunoglobulins and AB-AP using a

red chromogen (**3.3.2**). Molecular weight markers were run in an adjacent lane to calculate the weight of the detected proteins. Ovine luteal cell conditioned medium was used as a positive control for TIMP-1 (**3.1.1**). The intensity of each band was determined by computer aided densitometric image analysis (NIH Image 1.55) after image capture.

10.3.4 Northern Blotting

Thirty micrograms of total RNA was denatured, electrophoresed in a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane and fixed by u.v. cross-linkage (**3.6.1**). Membranes were pre-hybridised for 5 hours in hybridisation buffer (**3.6.2**). The human TIMP-1 cDNA probe (**3.1.3**) was labelled with 50 μCi ^{32}P dCTP by the random priming method (**3.5.5**). Hybridisation was performed at 65 °C for 20 hours (**3.6.2**). The membranes were washed (**3.6.3**), laid down to a phosphor screen for 24 hours and developed and quantified using a phosphorimager computer (**3.6.3**). To correct for minor differences in RNA loading the blots were stripped (**3.6.4**) and reprobed in the same manner with a cDNA probe to human β-actin (**3.1.3**). We found no differences in the level of β-actin expression during the different stages of the luteal phase. The molecular size of the transcripts was determined by running RNA markers in an adjacent lane (**3.6.1**). The ratio of relative intensities for TIMP-1 to β-actin, after normalisation by logarithmic transformation, was used for data analysis.

10.3.5 *In situ* Hybridisation

In situ hybridisation was performed on frozen sections using an antisense TIMP-1 ^{35}S-labelled riboprobe (**3.7**). The antisense probe was generated from the vector (**3.1.3**) linearised by BamH1 (**3.5.3**) using T7 RNA polymerase (**3.7.2**). The sense probe, which was used as the negative control, was generated from the vector (**3.7.1**) linearised by EcoR1 (**3.5.3**) using SP6 RNA polymerase (**3.7.2**). Frozen sections (6 μm) on poly-L-lysine-coated slides (**3.2.1**) were initially prepared as described previously (**3.7.3**). After acetylation the sections were treated using a different *in situ* hybridisation protocol than that described. In this case, the sections were washed in 2x SSC before dehydration and delipation through graded alcohols and chloroform.

The slides were air dried and incubated with pre-hybridisation buffer [50% formamide, 10 mM DTT, 1x Denhardt's solution, 4x STE (1x STE is 0.1 M NaCl,

10 mM Tris HCl pH 8, 1 mM EDTA), 125 μg/ml yeast tRNA, 125 μg/ml salmon sperm DNA] for one hour at 45 °C. Excess pre-hybridisation buffer was removed and slides were incubated with 1 x 10^6 c.p.m. of radio-labelled probe in 40 μl of hybridisation buffer [pre-hybridisation buffer containing 10% (v/v) dextran sulphate], sealed under hydrophobic film (**3.7.4**) and incubated overnight at 45 °C in a moist chamber. Sections were washed in 2x SSC/ 2 mM DTT prior to RNAse A treatment (30 μg/ml in RNAse buffer [10 mM Tris, 1 mM EDTA, 0.5M NaCl, pH 8]) for 30 min at 37 °C. The sections were sequentially washed in RNAse buffer/ 1 mM DTT and 2x SSC/ 1 mM DTT at room temperature before washing in 0.1x SSC/ 1 mM DTT at 45 °C in a shaking water bath. Sections were rinsed in 0.1x SSC then dehydrated through graded alcohols containing 0.3 M ammonium acetate and air-dried.

The sections were then dipped in photographic emulsion (**3.7.6**) and incubated in light-tight boxes at 4 °C for 21 days (**3.7.6**). The slides were then developed, fixed, counterstained with haematoxylin and mounted (**3.2.8**). The distribution of grains was analysed by dark field microscopy, after image capture, using a computer based image analysis system (Cue-2). The cellular composition of each field was identified by light field microscopy.

10.4 Results

10.4.1 Plasma Progesterone Concentrations

Classification of the corpora lutea by serial urinary LH measurement agreed with luteal phase dating of endometrial biopsies using the method of Li *et al.* (1988) in all cases. Plasma progesterone concentrations were 36.36 ±9.28 nmol/l for the early luteal tissue, 40.35 ±9.88 nmol/l for the mid luteal samples and 18.80 ±12.81 nmol/l for the late luteal samples. After luteal 'rescue' by exogenous hCG the plasma progesterone concentrations had increased to 52.75 ±1.09 nmol/l.

10.4.2 Immunohistochemistry

Immunostaining for TIMP-1 was present in corpora lutea of all stages of the luteal cycle and after luteal 'rescue'. As can be seen in Fig. 10.1*a*, staining was localised to the steroidogenic cells of the gland and absent from the negative controls (Fig. 10.1*b*). There were no differences in the localisation of immunostaining for TIMP-

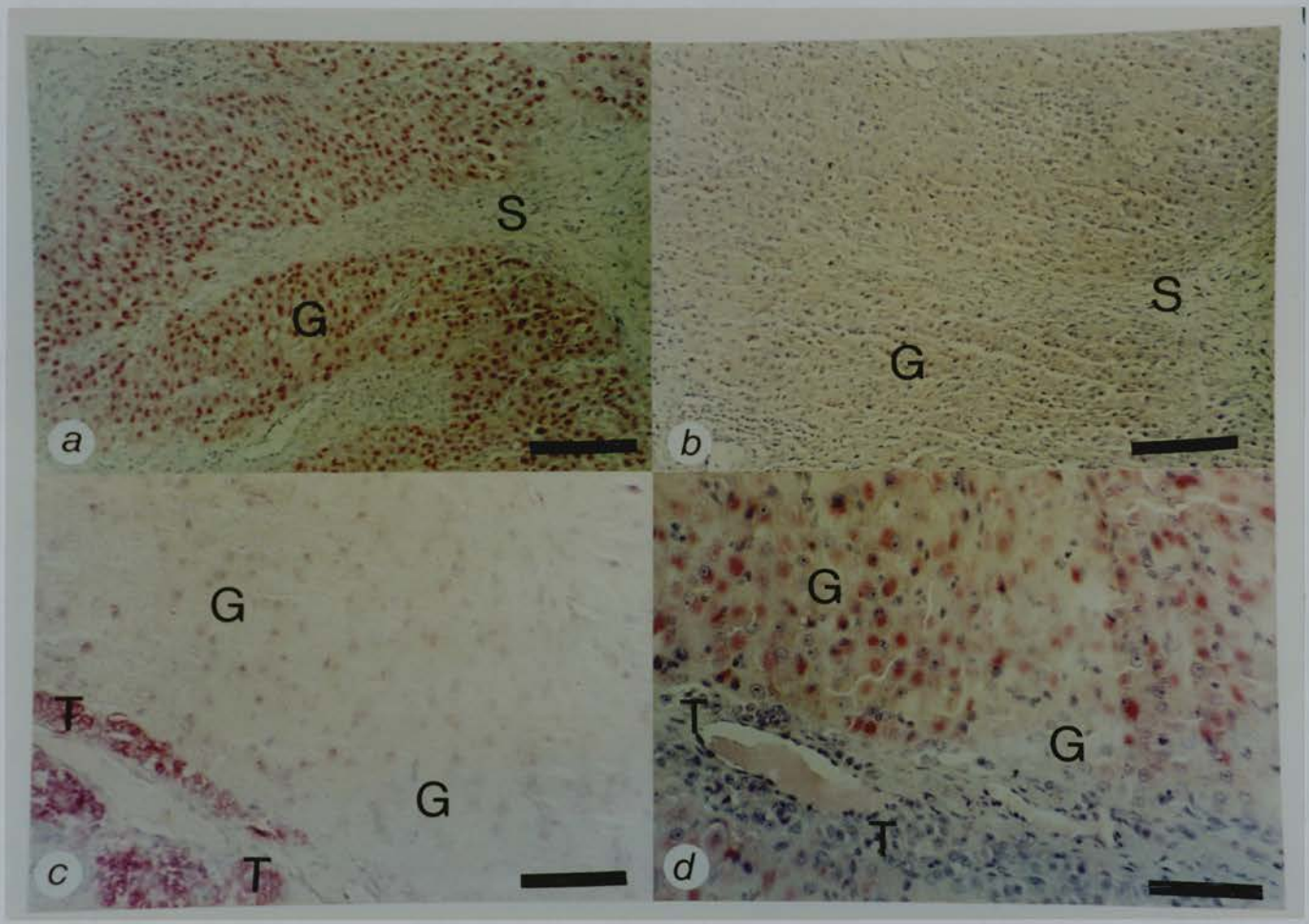


Figure 10.1

TIMP-1 immunostaining in human corpora lutea

Immunostaining of human corpora lutea: **a)** TIMP-1 protein in the early corpus luteum (red), staining is present in the steroidogenic cells (G) and absent from the surrounding stroma (S) (Scale bar = 100 μm); **b)** negative control serial section of (a) showing no staining of the steroidogenic cells (G) and the stroma (S) (Scale bar = 100 μm); **c)** Immunostaining for 17α-hydroxylase enzyme in a late-luteal corpus luteum (red). The theca-lutein cells are seen in discrete clumps (T) at the periphery of the granulosa-lutein cells (G); (Scale bar = 50 μm); **d)** Serial section of (c) stained for TIMP-1. The theca-lutein cells (T) are relatively free from staining and the granulosa-lutein cells (G) show specific TIMP-1 immunostaining (red); (Scale bar = 50 μm).

1 during the luteal cycle or after luteal 'rescue'. In particular, the regressing corpus luteum of the late-luteal phase showed no local areas of altered TIMP-1 immunostaining within the tissue section. The intensity of staining in all sections was classified as ++ or +++ with no obvious pattern of variation throughout the luteal cycle or after luteal 'rescue'. The steroidogenic cells derived from the theca were identified by immunostaining serial sections for the enzyme 17α-hydroxylase. Fig. 10.1*c* shows the theca-lutein cells to be present in discrete clumps along the periphery of the granulosa-lutein cells. This pattern of staining is similar to that reported by Tamura *et al.* (1992). The theca cells were relatively free from immunostaining for TIMP-1 as can be seen in Fig. 10.1*d*.

10.4.3 Western blotting

Western blotting showed a single band at 30 kilodaltons (kDa) as shown in Fig. 10.2. This is consistent with the molecular weight of TIMP-1 which has previously been reported (Smith GW *et al.,* 1994), and the same band could be detected in ovine luteal cell conditioned medium known to contain TIMP-1. This protein was detected in protein samples from human luteal tissue from all stages of the cycle. The intensity of the detected protein band in luteal cell extracts was similar at all stages of the luteal cycle and after luteal 'rescue'. The width of the detected band suggested there may be a heterologous population of TIMP-1 proteins, with different glycosylation patterns. We however found no consistent differences in protein band width during the different stages of the luteal phase.

10.4.4 Northern Blotting

A single band approximately 0.9 kb in length was detected in total RNA extracted from human corpora lutea by northern blotting. This size is consistent with TIMP-1 message reported in humans (Rapp *et al.*, 1990) and sheep (Smith GW *et al.,* 1994). As shown in Fig. 10.3, TIMP-1 mRNA was abundant and could be found at all stages of the luteal cycle and after luteal 'rescue'. Densitometric quantification of the intensity of the message standardised for tissue β-actin expression demonstrated no differences of level of TIMP-1 message during the luteal cycle and after luteal 'rescue' (Fig. 10.4).

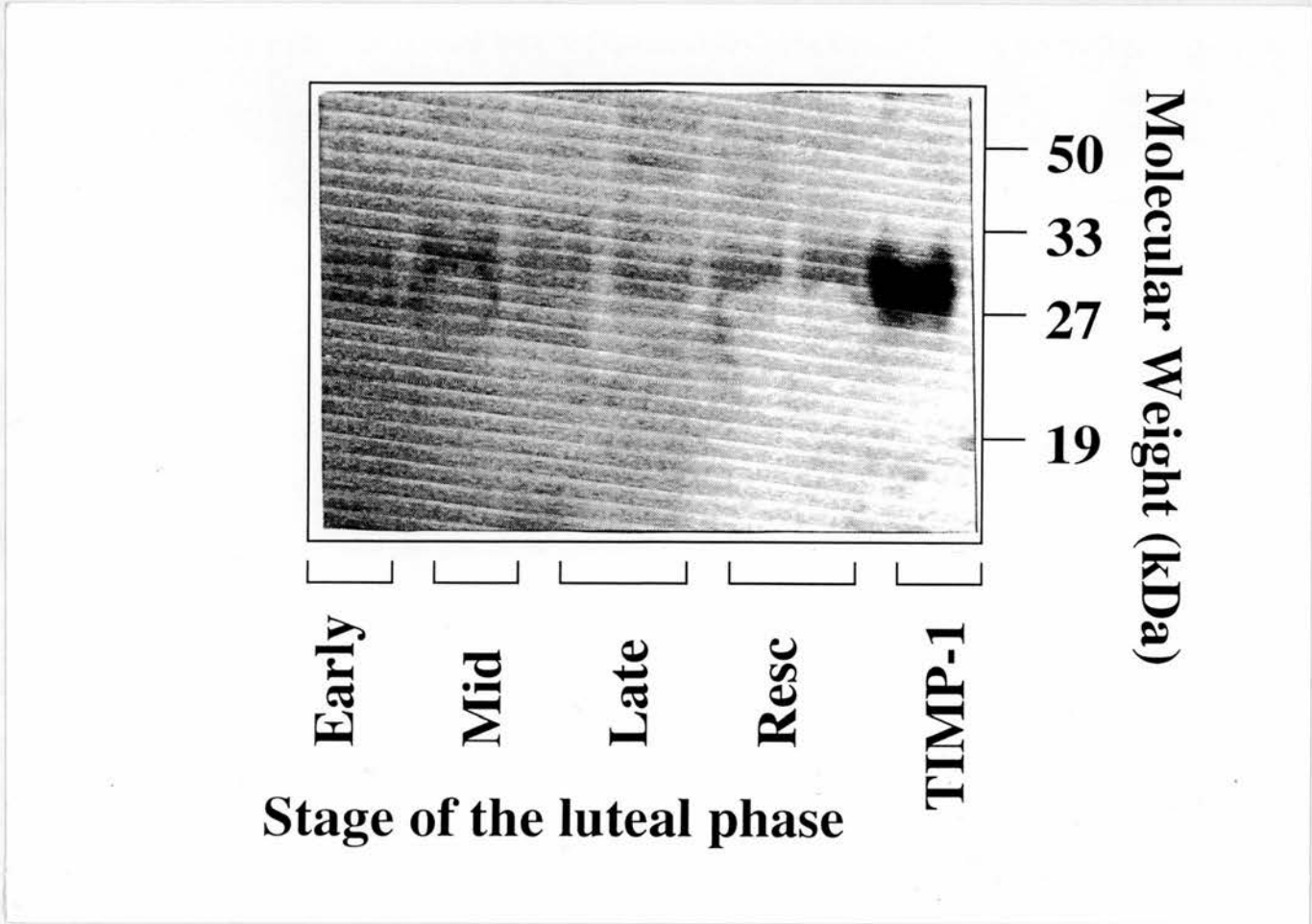


Figure 10.2

Western blot for TIMP-1 in human corpora lutea

Western blot for TIMP-1 protein in luteal tissue homogenates from different stages of the luteal phase and after luteal 'rescue' by exogenous hCG. Ovine luteal cell conditioned medium known to contain large quantities of TIMP-1 was used as a positive control. The migration of molecular weight markers is indicated at the right of the figure.

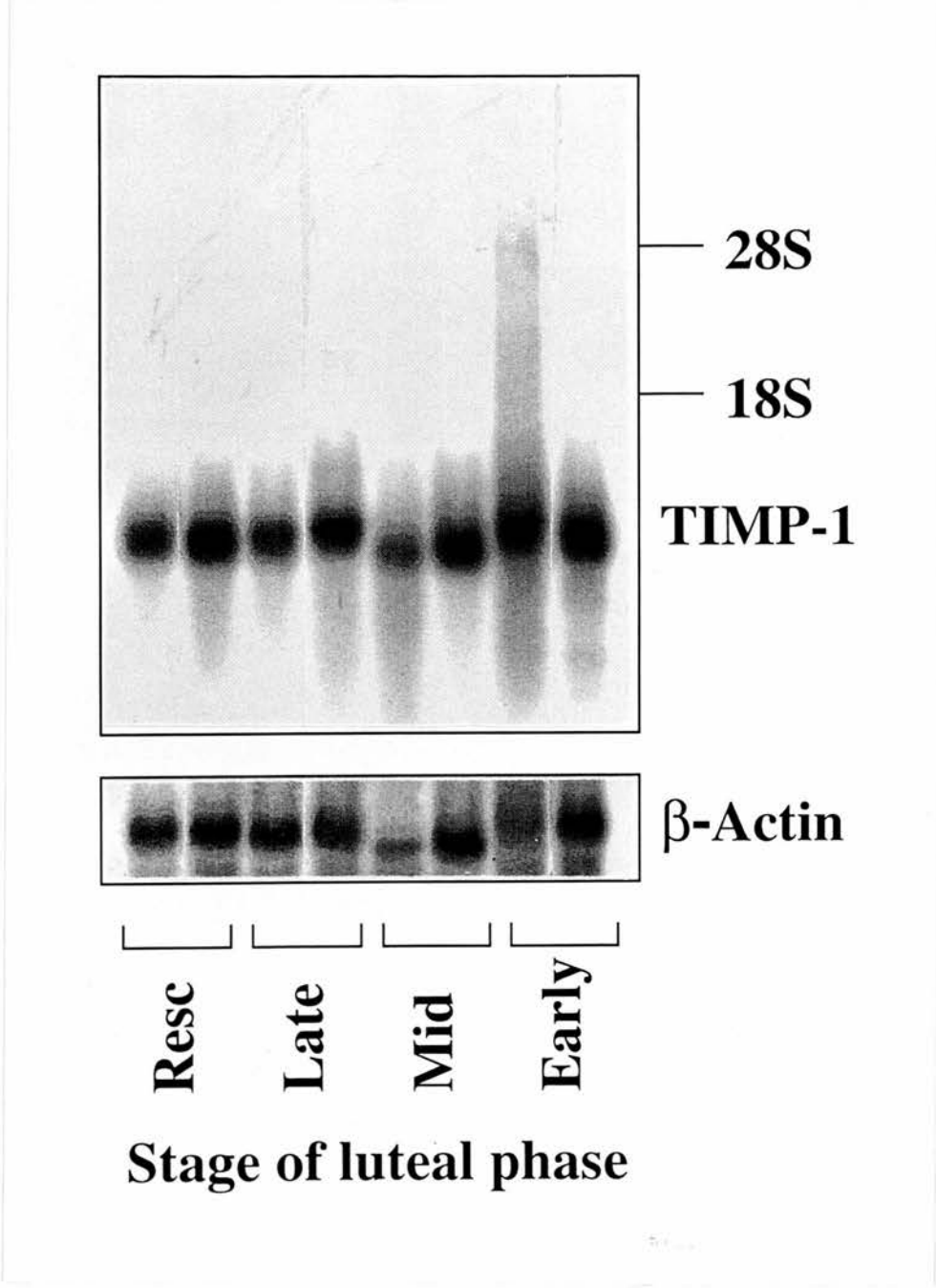


Figure 10.3

Northern blot of TIMP-1 mRNA in human corpora lutea

Northern blot of TIMP-1 mRNA using total RNA extracted from corpora lutea at different stages of the luteal phase and after luteal 'rescue' by exogenous hCG. The positions of the ribosomal 28S and 18S bands are indicated. The control bands detected after stripping the blot and re-probing for β-Actin are indicated below the TIMP-1 blot.

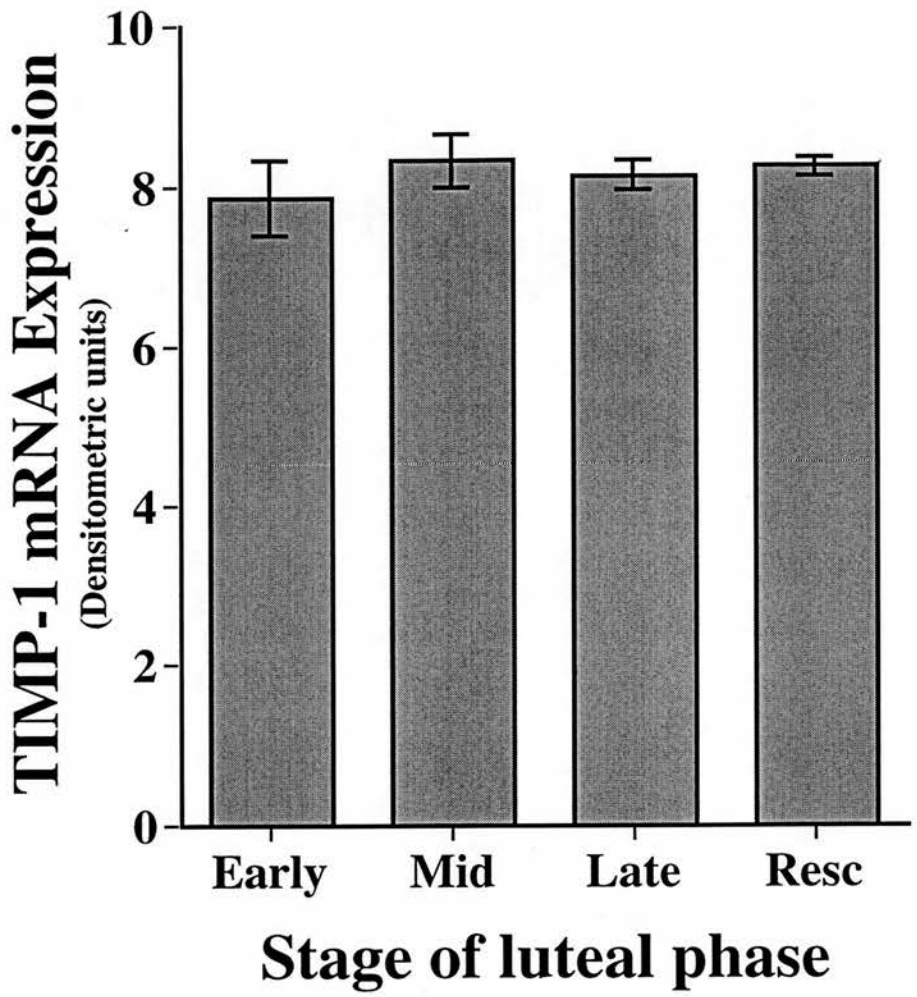


Figure 10.4

Expression of TIMP-1 mRNA in human corpora lutea

TIMP-1 mRNA in corpora lutea from different stages of the luteal phase and after luteal 'rescue' by exogenous hCG. Message for TIMP-1 detected by northern blotting was quantified by computer-aided densitometry corrected for minor loading variations by comparison of β-actin levels.

10.4.5 *In situ* Hybridisation

As shown in Fig. 10.5*a*, message for TIMP-1 was uniformly distributed throughout the granulosa-lutein cells of the corpus luteum. This distribution was only seen in the sections incubated with the antisense probe and was absent from control sections incubated with the sense probe (Fig. 10.5*b*). The localisation of TIMP-1 mRNA in luteal cells is consistent with the localisation of TIMP-1 protein (Fig. 10.1*a*). Luteal cells of thecal origin had much lower grain concentrations than those granulosal cell origin (Fig. 10.5*c,d*). The localisation of TIMP-1 mRNA did not change throughout the luteal cycle or after luteal 'rescue'.

10.5 Discussion

This study is the first to demonstrate the expression and localisation of TIMP-1 in the human corpus luteum. TIMP-1 mRNA has previously been demonstrated in the corpus luteum of a number of other species including the sheep (Smith GW *et al.*, 1994), the cow (Juengel *et al.*, 1994) and the pig (Smith MF *et al.*, 1994). Indeed it has previously been shown that TIMP-1 is one of the major products of the ovine corpus luteum, and our data suggest that TIMP-1 is similarly abundant in the luteal cells of the human ovary. The immunohistochemistry and immunoblotting demonstrate specific binding to a protein of the appropriate molecular weight while the Northern analysis and *in situ* hybridisation experiments confirmed the source of specific mRNA expression.

The TIMP-1 protein and mRNA were found to be present specifically in the granulosa-lutein cell-types of the corpus luteum. Human pre-ovulatory granulosa cells have previously been shown to produce metalloproteinase inhibitor activity *in vitro* (Morgan *et al.*, 1994) and contain abundant TIMP-1 mRNA (Rapp *et al.*, 1990). TIMP-1 is expressed in the granulosa cells of the pre-ovulatory follicle in the sheep at a 15 fold higher level than in the theca cells of the same follicle (Smith GW *et al.*, 1994). We identified the steroidogenic cells of thecal origin in the corpus luteum by immunostaining with an antibody to 17α-hydroxylase. This enzyme is not present in the granulosa-lutein cells and has been used to identify cells of thecal origin in human corpora lutea (Tamura *et al.*, 1992; Rodger *et al.*, 1995). We found that there was little if any immunostaining, or *in situ* hybridisation for TIMP-1 in the theca-lutein cells of the human corpus luteum. As the localisation of TIMP-1 appeared to be confined to the large steroidogenic cells

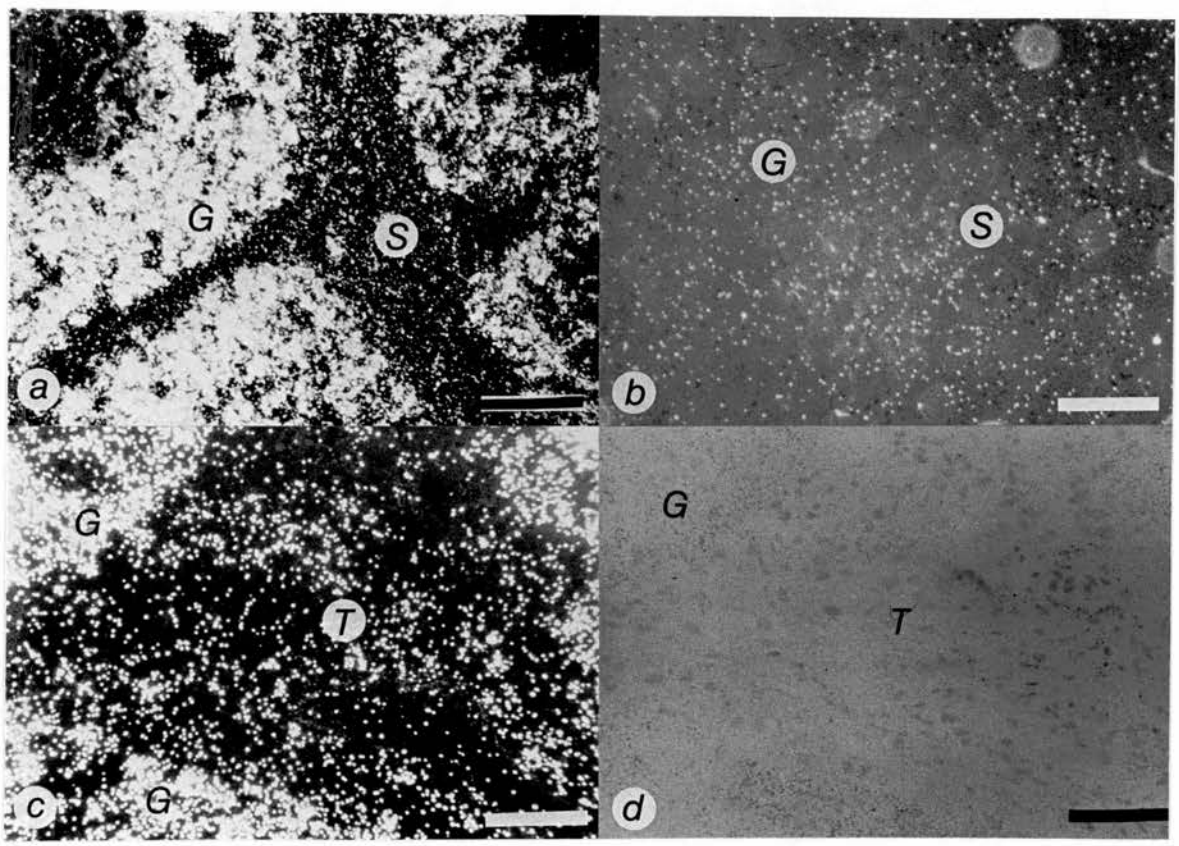


Figure 10.5

***In situ* hybridisation of TIMP-1 mRNA in human corpora lutea**

In situ hybridisation of human corpora lutea for TIMP-1 mRNA: **a)** dark-field of TIMP-1 mRNA in the early corpus luteum. Grains are found in the steroidogenic cells (G) at much higher levels than the surrounding stroma (S); (Scale bar = 100 μm); **b)** dark-field negative control serial section of (a) using sense TIMP-1. There are no differences in grain localisation in the steroidogenic cells (G) and the stroma (S); (Scale bar = 100μm); **c)** dark-field of luteal TIMP-1 mRNA showing uneven distribution of grains within the steroidogenic cell layer (Scale bar = 50 μm); **d)** light-field of (c) showing the granulosa-lutein cells (G) and the theca-lutein cells (T). Grains are present at much higher levels in the granulosa-lutein cells (G) than the theca-lutein cells (T); (Scale bar = 50 μm).

of the corpus luteum it is unlikely that non-steroidogenic cells present in the corpus luteum such as macrophages, T-lymphocytes (Brännström *et al.*, 1994a) and endothelial cells (Rodger *et al.*, 1997) contribute to TIMP-1 production, but this remains to be established.

No change was found in either the site or intensity of expression of TIMP-1 during the luteal phase or following luteal 'rescue' with hCG. This was surprising as luteal expression of TIMP-1 has previously been shown to rise for up to eight hours during $PGF_{2\alpha}$ induced luteolysis in the cow (Juengel *et al.*, 1994). The same group localised TIMP-1 mRNA by *in situ* hybridisation during luteolysis and found that scattered individual cells within the steroidogenic tissue expressed much higher levels than the surrounding cells (Smith GW *et al.*, 1994). This pattern of expression was unlike the uniform grain distribution seen within ovine follicular granulosa cells (Smith GW *et al.*, 1994). In the human corpus luteum we found that expression of TIMP-1 is uniform in the granulosa-lutein cell population and this distribution is maintained throughout the luteal cycle. The different patterns of expression are probably due to a more gradual luteolytic process in women. Although we studied four corpora lutea from the late-luteal phase, all the corpora lutea studied were still functional, in that progesterone secretion was continuing. Unlike the ruminant, luteolysis in women does not result from a surge of uterine prostaglandin and is therefore likely to be a more gradual process (Auletta and Flint, 1988). In contrast in cattle, prostaglandin treatment produces co-ordinated luteolysis which allows changes induced in a proportion of cells, such as apoptosis, to be detected more easily (Juengel *et al.*, 1993). The more gradual fall in luteal function in women may mask the increased TIMP-1 expression seen within individual cells during luteolysis in the ovine gland.

Luteal involution is inhibited during maternal recognition of pregnancy by hCG acting through the LH receptor (Behrman *et al.*, 1993). Expression of TIMP-1 in preovulatory granulosa cells increases during luteal formation at the time of the gonadotrophin surge (Smith GW *et al.*, 1994). At this stage the granulosa cells contain LH receptors (Richards and Midgley, 1976), and it is possible that the increase in TIMP-1 is partially mediated through stimulation of this receptor. In the rat, LH stimulates a TIMP-like protein in granulosa cell culture media and TIMP-1 mRNA is increased by LH (Mann *et al.*, 1991; Morgan *et al.*, 1994). However, it does not appear that further stimulation of the corpus luteum by hCG, as occurs in normal pregnancy, provokes any further increase in TIMP-1

synthesis. This may be a species-related difference as Morgan *et al.* (1994) were unable to show any increase in TIMP-1 in response to LH in cultured human granulosa cells. TIMP-1 expression in women may be controlled by other hormones such as progesterone. Progesterone receptors have been identified in the human corpus luteum (Suzuki *et al.*, 1994) and the progesterone antagonist RU486 can reduce TIMP activity in granulosa cell cultures (Morgan *et al.*, 1994).

The significance of the TIMP-1 produced by the human corpus luteum remains unclear but its production in large quantities throughout the luteal cycle suggests an important role. In view of this, Juengel *et al.*, (1994) have suggested that TIMP-1 expression reflects the production of endogenous MMPs and acts as a protective mechanism to limit specific protease action. However, as described in this paper, TIMP-1 mRNA is abundant in the corpus luteum. It is one of the most abundant mRNAs in luteinised granulosa cells (Rapp *et al.*, 1990) and TIMP-1 is the major protein product of the ovine corpus luteum (Smith *et al.*, 1993). In contrast, recent results suggest that the mRNAs for MMPs are present in far lower concentrations than that of TIMP-1 (Nothnick *et al.*, 1995; Nothnick *et al.*, 1996). As TIMP-1 binds to and inactivates MMPs in a 1:1 ratio (Matrisian, 1990) it is probable that in the corpus luteum, MMPs are functioning in an environment containing a high level of specific inhibitor. For MMPs to function in this environment it is likely that the interaction between MMPs and TIMPs is at a local cellular rather than a tissue-wide level. It may be that local tissue remodelling is regulated by the expression of proMMPs and their subsequent activation to active MMPs, rather than control of inhibition. It is consequently difficult to assess the function of TIMP-1 in isolation, when the activity and production of MMPs and other inhibitors such as TIMP-2 remain unclear during the luteal cycle, particularly as it has recently been reported that TIMP-2 is also produced by the ovine corpus luteum (Smith *et al.*, 1995a).

TIMP-1 has other properties which may contribute to a critical role in maintenance of the corpus luteum as well as the inhibition of metalloproteinase activity. TIMP-1 promotes the proliferation of fibroblasts and endothelial cells and may be a paracrine or autocrine growth factor (Hayakawa *et al.*, 1992). This may be important to facilitate the high levels of angiogenesis during corpus luteum formation (Reynolds *et al.*, 1992). It is also possible that TIMP-1 has a role in cell migration (Smith GW *et al.*, 1994) or protection against neoplastic stimuli during periods of tissue growth and neovascularisation. A recent publication has raised

the possibility that TIMP-1 particularly in combination with procathepsin-L may be involved in regulating steroidogenesis (Boujrad *et al.*, 1995). Boujrad *et al.*, (1995) found that a locally produced FSH-responsive factor which stimulates both Leydig and granulosal cell steroidogenesis is the TIMP-1-procathepsin-L complex. Both TIMP-1 and this complex were found to stimulate steroidogenesis in a cAMP-independent manner with a bioactivity similar to saturating amounts of hCG. Although the effect of TIMP-1 and the procathepsin-L complex on luteal steroidogenesis are not yet known, it is notable that the corpus luteum has a very high level of synthesis of both TIMP-1 and steroids.

In summary, TIMP-1 is an abundant secretory product of the human corpus luteum during both the normal menstrual cycle and in simulated early pregnancy. The physiological role of the TIMP-1 is unclear but may involve the facilitation of steroidogenesis and the protection of the ovary against the effects of local metalloproteinase production.

Chapter 11

Expression of TIMP-1 in the primate ovary during induced luteal regression

11.1 Abstract

Although TIMP-1 is one of the major secretory products of the corpus luteum, the functional significance of this is not clear. In addition to its role as a specific inhibitor of the MMPs involved in tissue remodelling, it has recently been suggested that TIMP-1 is also a potent stimulator of steroidogenesis *in vitro*. However, in the ruminant, TIMP-1 expression increases during luteal regression. This study sought to determine (i) the effect of induced luteal regression on ovarian TIMP-1 expression in the primate and (ii) the expression of TIMP-1 in other steroidogenic and non-steroidogenic tissues. Marmoset ovaries were studied on day 10 of the normal luteal phase, and 12 and 24 hours after induced luteolysis, with either $GnRH_{ant}$ or $PGF_{2\alpha}$ analogue. Ovaries from different stages of the normal ovarian cycle were also studied. Expression of TIMP-1 was investigated by isotopic *in situ* hybridisation. TIMP-1 expression was also examined in a wide range of other marmoset tissues by northern blotting and *in situ* hybridisation. TIMP-1 was found to be highly expressed in the marmoset corpus luteum. Induced luteolysis, with either $PGF_{2\alpha}$ or $GnRH_{ant}$, was associated with a significant fall in TIMP-1 expression in luteal tissue. TIMP-1 mRNA was also localised to ovarian follicles throughout the ovarian cycle. Expression occurred in the thecal layer of smaller follicles (<1.5 mm) and the granulosal layer of larger preovulatory follicles. In atretic follicles, TIMP-1 was highly expressed at the interface between the thecal and granulosal cells. TIMP-1 was found to be predominantly expressed in steroidogenic tissues, particularly the ovary, adrenal and placenta. These data support a role for changes in TIMP-1 expression in tissue remodelling in the ovary and are consistent with an additional function of TIMP-1 as a facilitator of steroidogenesis.

11.2 Introduction

Unless chorionic gonadotrophin is produced by the implanting blastocyst, the primate corpus luteum will stop secreting progesterone and become a small fibrous remnant. The mechanisms of functional and structural luteolysis in the primate are still poorly understood (Behrman *et al.*, 1993). The highly vascular corpus luteum is formed from the dominant follicle and in turn becomes the avascular corpus albicans in a matter of weeks. This process involves extensive tissue remodelling (Luck and Zhao, 1995). The MMPs are a group of zinc-dependent proteolytic enzymes which have been implicated in remodelling of the ECM (Birkedal-Hansen, 1995). The activity of these enzymes is controlled at several levels, including their synthesis as pro-enzymes, enzyme activation, and the production of specific inhibitors (Matrisian, 1990). The corpus luteum is known to produce specific TIMPs (Smith *et al.*, 1993; Smith *et al.*, 1995a). One of these, TIMP-1, is of particular interest as it is one of the major products of the corpus luteum of many species, including the sheep (Smith *et al.*, 1993), cow (Jeungel *et al.*, 1994), pig (Smith MF *et al.*, 1994) and, as shown in **Chapter 10**, the human (Duncan *et al.*, 1996c).

The role of TIMP-1 in the corpus luteum has yet to be elucidated. Jeungel *et al.* (1994) found that the expression of TIMP-1 increased after $PGF_{2\alpha}$-induced luteolysis in the cow, and postulated that it may have an important role in tissue remodelling during luteolysis. However, it is not known whether TIMP-1 expression is increased during luteolysis in the primate. We did not observe any change in TIMP-1 expression over the functional lifespan of the human corpus luteum (Duncan *et al.*, 1996c), implying that TIMP-1 may have other functions in the primate corpus luteum. Boujrad *et al.* (1995) found that a locally produced FSH-responsive factor, which stimulated both Leydig and granulosal cell steroidogenesis, was the TIMP-1-procathepsin-L complex. Both TIMP-1 and this complex were found to stimulate steroidogenesis in a cAMP-independent manner with a bioactivity similar to saturating amounts of hCG. It is therefore possible that one role of TIMP-1 is to facilitate steroidogenesis.

This study aimed to investigate the expression of TIMP-1 during luteolysis in the primate corpus luteum. The marmoset monkey was used as a model, as luteolysis can be induced by both $PGF_{2\alpha}$ and LH withdrawal (Fraser *et al.*, 1995b). As whole primate ovaries were studied, we were also able to describe the localisation

of TIMP-1 mRNA in ovarian follicles. In order to investigate the possibility of a general role for TIMP-1 in steroidogenesis, TIMP-1 expression was studied in a wide range of endocrine and non-endocrine tissues.

11.3 Specific Materials and Methods

11.3.1 Tissues Studied

Captive-bred common marmoset monkeys were studied (**2.3.1**). To confirm normal ovulatory cycles, plasma samples were assayed for progesterone to determine the date of ovulation and the luteal phase duration (**2.3.1**). Ovaries were collected on day 10 of the luteal phase (**2.3.3**). Ovaries were collected from untreated control animals (n=4) and animals treated with either $PGF_{2\alpha}$ analogue (**2.3.2**), 12 hours (n=3) or 24 hours (n=3) previously or $GnRH_{ant}$ (**2.3.2**), 12 hours (n=3) or 24 hours (n=3) previously. Whole ovaries were fixed in 4% paraformaldehyde and embedded in paraffin wax (**2.3.3**). In addition, some marmoset ovaries were available which had been frozen in embedding medium after removal (**2.3.3**). These included follicular phase ovaries (n=4), luteal phase ovaries (n=3), and other ovaries where luteolysis had been induced as described above (n=8).

A bank of normal marmoset tissues, collected from this and other experiments, was also utilised (**2.3.3**). These tissues were removed immediately post-mortem. A piece of each tissue was snap frozen in liquid nitrogen and stored at -70 °C for subsequent RNA extraction. Another piece was frozen in embedding medium, and stored at -70 °C until frozen sections were prepared (**2.3.3**).

11.3.2 *In situ* Hybridisation

Isotopic *in situ* hybridisation was performed on both fixed and frozen sections using ^{35}S-labelled riboprobes (**3.7**). Antisense and sense riboprobes, incorporating ^{35}S-labelled UTP, were generated (**3.7.2**) from the full-length human TIMP-1 cDNA construct (**3.1.3**). The antisense probe was generated from the plasmid vector (**3.7.1**) linearised by Kpn1 (**3.5.3**) using T7 RNA polymerase (**3.7.2**). The sense probe was used as the negative control. This was generated from the plasmid vector (**3.1.3**) linearised by HindIII (**3.5.3**) using SP6 RNA polymerase

(**3.7.2**). Sections of mid-luteal human corpus luteum (**2.2**) were used as a positive control in each experiment.

Fixed sections (5 μm) on poly-L-lysine-coated slides (**3.2.1**) were prepared (**3.7.3**) and incubated with proteinase K (**3.7.3**). Frozen sections were quickly thawed and fixed in 4% paraformaldehyde (**3.7.3**). All slides were then washed, acetylated, dehydrated and dried under vacuum (**3.7.3**). One hundred microlitres of hybridisation buffer (**3.7.4**) containing 1 x 10^6 c.p.m. radiolabelled probe was added to each section. The slides were covered with a hydrophobic coverslip and hybridised overnight at 55 °C in a moist chamber (**3.7.4**).

The coverslips were washed off (**3.7.5**) and the slides were treated with RNAse A (**3.7.5**). After increasingly stringent washes (**3.7.5**), the slides were washed for 30 minutes in 0.1x SSC at 70 °C (**3.7.5**). They were then dehydrated through graded alcohols and allowed to dry (**3.7.5**). The slides were then dipped in photographic emulsion (**3.7.6**) and stored at 4 °C for 18 days in the dark. After developing and fixing (**3.7.6**), the slides were washed, counterstained in haematoxylin and mounted (**3.2.8**).

11.3.3 Northern Blotting

Twenty micrograms of total RNA (**3.6.1**) was denatured, electrophoresed in a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane (**3.6.1**) and fixed to the membrane by u.v. cross-linkage (**3.6.1**). After pre-hybridisation for two hours (**3.6.2**), the membranes were hybridised for 20 hours at 65 °C (**3.6.2**) with a full-length human TIMP-1 cDNA probe (**3.1.3**) labelled with 50 μCi ^{32}P dCTP (**3.5.5**). After washing (**3.6.3**) the blots were laid down to a phosphor screen for 48 hours and visualised using a phosphorimager computer (**3.6.3**). To confirm accurate loading of RNA, the blots were stripped (**3.6.4**) and reprobed with a ^{32}P end-labelled oligonucleotide (**3.1.3**) which hybridises to 18S RNA (**3.6.4**).

11.3.4 Analysis of Results

Sections were viewed by dark-field microscopy and analysed after image capture by computer-aided image analysis (NIH Image 1.55). To investigate the grain distribution over the corpus luteum, only fixed sections, which had undergone *in situ* hybridisation in carefully controlled conditions in the same run, were analysed. The corpora lutea in each section were identified and the grain density

in five fields were analysed by the same observer, blinded to the tissue identity, using a technique of stratified random sampling, and monitoring the running mean. Any acellular areas, or areas of the section without the corpus luteum were ignored. This was repeated at a later date by the same observer to confirm the reproducibility of the results. As TIMP-1 is highly expressed in the corpus luteum, the area proportion of grains was measured, after binary conversion, rather than the absolute grain count. Differences in the area proportion of grains between different groups were investigated by one way ANOVA. Where an overall statistically significant difference, at the 5% level, was detected, pairwise comparisons between groups were performed using Bonferroni/Dunn multiple range tests.

As TIMP-1 was also localised to the follicle, the expression of TIMP-1 was analysed in each follicle. All of the different ovarian sections, fixed and frozen, were analysed after *in situ* hybridisation. In order to obtain an idea of the localisation of TIMP-1 as the follicle developed, a technique was devised to classify individual follicles in accordance with their size. Each follicle was measured in light-field using an eyepiece graticule calibrated with a standard micrometer slide. The maximal dimension of the follicle from the outside edge of the thecal layer was calculated. As grains were only seen in follicles >200 μm, only these follicles were counted. These were classified as i) pre-antral, if there was no antrum visible in the plane of section, ii) antral, if an antrum was visible and the follicle measured <600 μm, iii) small, if the follicle measured <1 mm, iv) medium, if the follicle measured <1.5 mm and v) large, if the follicle measured 1.5 mm or greater. The appearance of each follicle was then classified by morphology as normal or atretic (Grimes *et al.*, 1987). The grain distribution over the thecal and granulosal layer of each follicle was observed in dark-field of the same slides and recorded as absent or present.

11.4 Results

11.4.1 Plasma Progesterone Concentrations

Progesterone concentrations in the control animals were 330 ±69 nmol/l (mean ±S.E.M.). Functional luteal regression was observed in all animals treated with either the $GnRH_{ant}$ or the $PGF_{2\alpha}$ analogue, as described previously (Fraser *et al.*,

1995b). $PGF_{2\alpha}$ treatment resulted in a decline in progesterone concentrations to 20 ±5 nmol/l after 12 hours and 22 ±6 nmol/l after 24 hours, and treatment with $GnRH_{ant}$ resulted in progesterone concentrations of 13 nmol/l after 12 hours and 23 ±11 nmol/l after 24 hours. All progesterone concentrations after induced luteolysis were within the normal range of follicular phase levels in the marmoset (Smith *et al.*, 1990).

11.4.2 TIMP-1 in the Corpus Luteum

TIMP-1 mRNA was expressed in the granulosa-lutein cells of the human corpus luteum (Fig. 11.1*a*). The human corpus luteum had been included as a positive control as it expresses large amounts of TIMP-1 (Duncan *et al.* 1996c). This grain distribution was absent from all negative control sections, which had been incubated with the sense probe (Fig. 11.1*b*).

TIMP-1 message was localised to the corpus luteum in luteal-phase marmoset ovaries (Fig. 11.1*c,d*). TIMP-1 mRNA could also be localised to the cells of the corpora lutea, 12 and 24 hours after induced luteolysis (Fig. 11.1*e,f*). However, the silver grains over the corpus luteum were lower in number and more patchily distributed after induced luteolysis (Fig. 11.1*e,f*). The area proportion of grains decreased significantly after both $PGF_{2\alpha}$ ($p<0.05$) and $GnRH_{ant}$ ($p<0.05$) treatments (Fig. 11.2). When specific time points were analysed, grain density was significantly lower 24 hours after $PGF_{2\alpha}$ treatment ($p<0.05$), and both 12 ($p<0.05$) and 24 hours ($p<0.01$) after $GnRH_{ant}$ treatment (Fig. 11.2). Treatment with $GnRH_{ant}$ or $PGF_{2\alpha}$ analogue produced similar changes in the appearance of the corpora lutea and the localisation of TIMP-1 mRNA.

11.4.3 TIMP-1 in the Follicle

TIMP-1 mRNA was also identified in ovarian follicles (Fig. 11.3*a,b*). TIMP-1 expression was absent from the oocyte, the primordial follicle and the small pre-antral follicle <200 μm in diameter. Follicles 200 μm or greater in diameter expressed TIMP-1 in the thecal cell layer (Fig. 11.3*a,b,c,d*). In the healthy follicles, TIMP-1 was absent from the granulosal cell layer at this stage. In larger pre-ovulatory follicles (≥1.5 mm) TIMP-1 was noted to be absent from the thecal layer and present in large amounts in the granulosal cell layer (Fig. 11.3*e,f*). When the follicle was atretic, a specific localisation of TIMP-1 was noted. TIMP-1

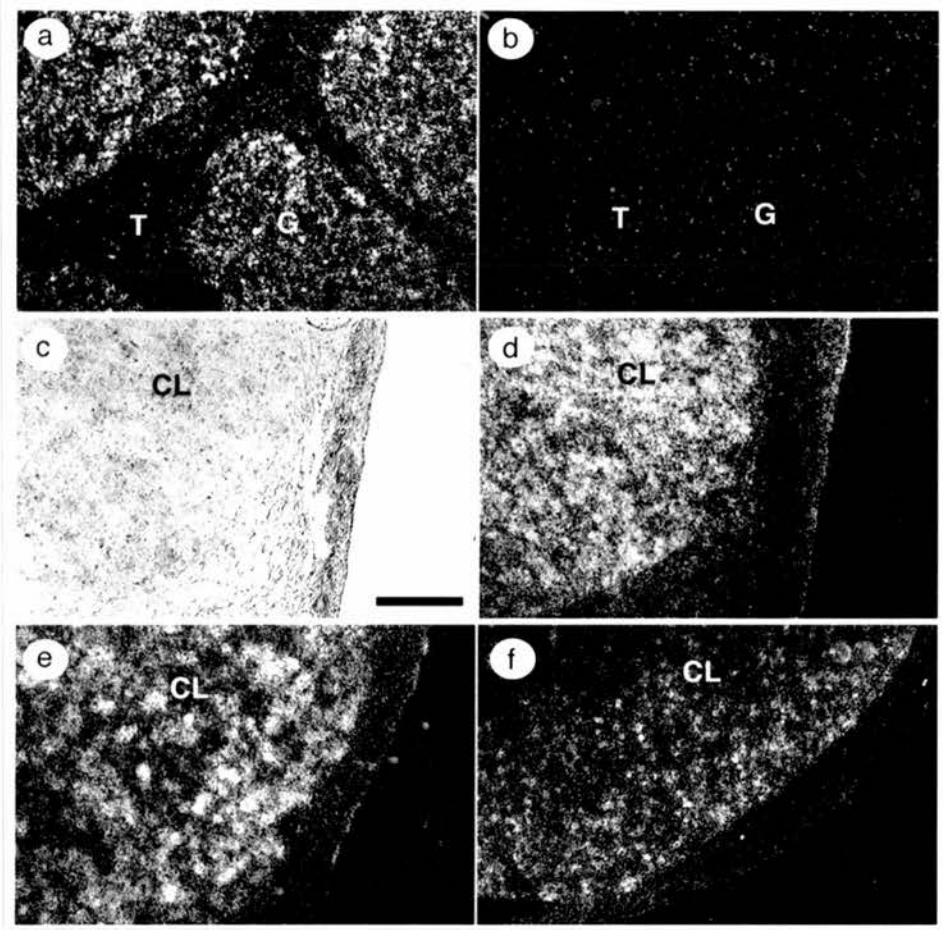


Figure 11.1

TIMP-1 mRNA in the marmoset corpus luteum

In situ hybridisation for TIMP-1 mRNA in the primate corpus luteum: **a)** dark-field of TIMP-1 mRNA in the mid-luteal human corpus luteum. Many more grains are seen over the granulosa-lutein cells (G) than the theca-lutein cells (T); **b)** dark-field negative control serial section of (a) showing very few grains with no difference between the granulosa-lutein cells (G) and the theca-lutein cells (T); **c)** light-field of a mid-luteal marmoset ovary showing the position of the corpus luteum (CL); **d)** dark-field of section (c) showing TIMP-1 grains localised to the corpus luteum (CL); **e)** dark-field of TIMP-1 mRNA in the corpus luteum (CL) of a marmoset ovary 12 hours after treatment with $GnRH_{ant}$; **f)** dark-field of TIMP-1 mRNA in the marmoset corpus luteum (CL) 24 hours after $GnRH_{ant}$ treatment. (Scale Bar = 200 μm).

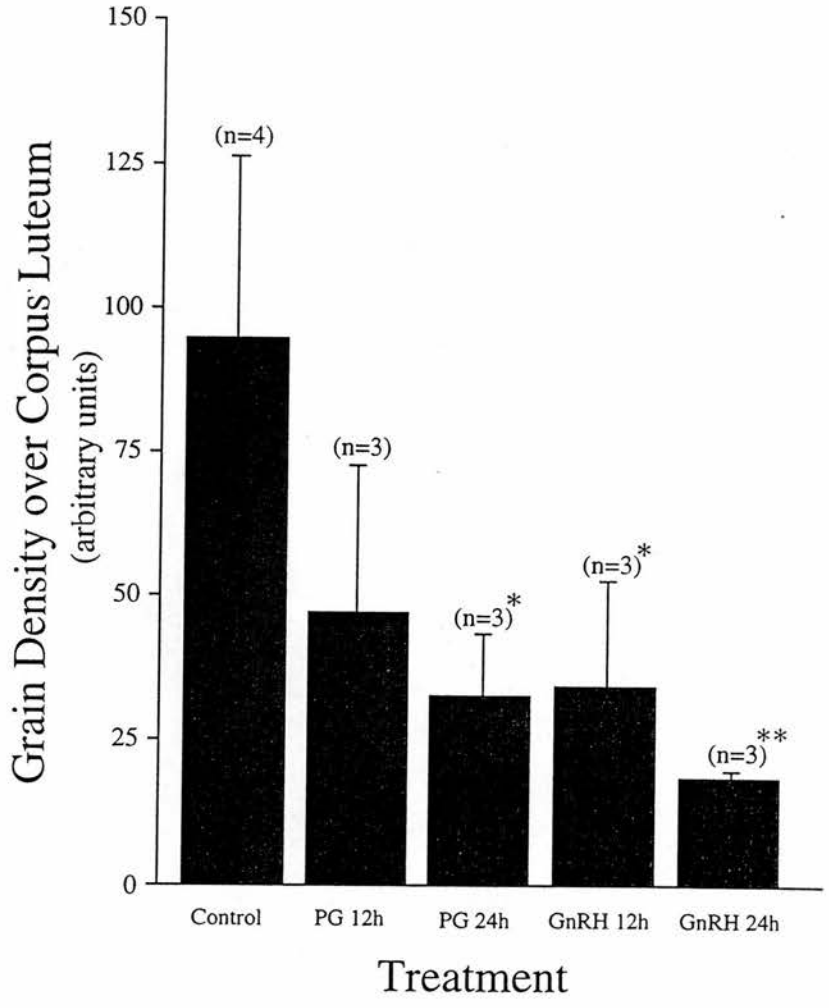


Figure 11.2

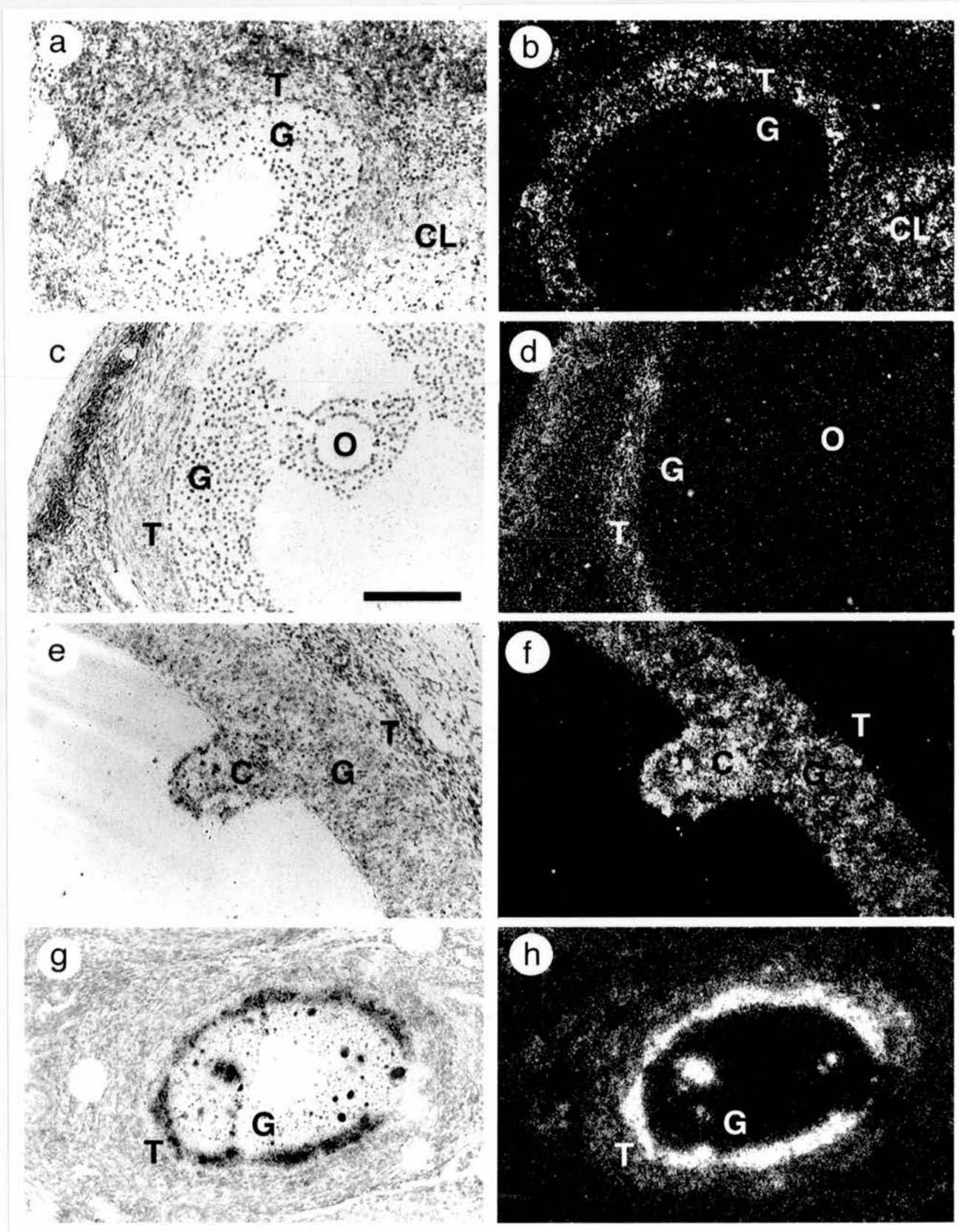
Expression of TIMP-1 mRNA after induced luteolysis

TIMP-1 mRNA in the marmoset corpus luteum after induced luteolysis as measured by grain density after *in situ* hybridisation. Luteolysis had been induced by $PGF_{2\alpha}$ analogue (PG) or $GnRH_{ant}$ (GnRH), 12 or 24 hours previously. Values are means ±S.E.M., significant differences from the control group are indicated for each experimental group: *=$p<0.05$; **=$p<0.01$.

Figure 11.3

TIMP-1 mRNA expression in follicles

In situ hybridisation for TIMP-1 mRNA in marmoset follicles: **a)** light-field of a normal luteal phase marmoset ovary showing a small follicle, with its granulosal (G) and thecal (T) layers next to a corpus luteum (CL); **b)** dark-field of section (a) showing localisation of TIMP-1 mRNA by *in situ* hybridisation. Grains are seen over the thecal cells (T) and corpus luteum (CL) but are absent from the granulosal cells (G); **c)** light-field of a follicular phase ovary showing a medium-sized follicle with the thecal cells (T), granulosal cells (G) and oocyte (O) clearly visible; **d)** dark-field of section (c) showing TIMP-1 localised to the thecal cells (T) but absent from the granulosal cells (G) and the oocyte (O); **e)** light-field of large pre-ovulatory follicle taken from another follicular phase marmoset ovary showing the thecal layer (T), the granulosal layer (G) and the cumulus granulosa cells (C) surrounding the oocyte; **f)** dark-field of section (e) showing TIMP-1 mRNA grains over the granulosa (G) and cumulus cells (C) but absent from the thecal cells (T); **g)** light-field of an atretic follicle in an ovary collected 12 hours after treatment with $PGF_{2\alpha}$ analogue, showing the thecal (T) and granulosal (G) layers; **h)** dark-field of section (g) showing some TIMP-1 expression in the thecal cells (T), patchy expression in the granulosal cells (G) and marked expression at the thecal-granulosal interface. (Scale bar = 200 μm).


a
T
G
CL
b
T
G
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mRNA was found to be localised to theca cells and individual granulosa cells, and was intensely expressed at the thecal-granulosal interface (Fig. 11.3*g,h*). The changing pattern of TIMP-1 localisation noted in the follicle was remarkably consistent in all the follicles analysed (Table 11.1). The pattern of TIMP-1 expression by the follicles of different sizes appeared to be independent of the stage of the cycle at which the ovary was obtained and was thus identical in ovaries obtained from the follicular phase, the luteal phase, or following induced luteolysis.

11.4.4 TIMP-1 in Other Tissues

TIMP-1 was found to be expressed in a wide range of marmoset tissues (Fig. 11.4). A single band, approximately 0.9 kb in length, was detected in total RNA extracted from marmoset tissues. This size is consistent with that reported for human TIMP-1 mRNA (Rapp *et al.*, 1990; Duncan *et al.*, 1996c). The highest levels of expression were seen in steroidogenic tissues, particularly the ovary, adrenal and placenta. Other tissues had low levels of TIMP-1 expression, although specific expression was noted in the thyroid and prostate glands. However, when the localisation of TIMP-1 message was investigated, in these tissues, by *in situ* hybridisation, the grain density was found to be much less than that seen in the ovary. It became apparent, using *in situ* hybridisation, that TIMP-1 was also expressed in the testis, specifically in the Sertoli cells and interstitial cells (Fig. 11.5*a,b*). Specific hybridisation was seen over the steroidogenic cells of the adrenal cortex (Fig. 11.5*c*), but not the adrenal medulla. Very few grains were seen over the kidney (Fig. 11.5*d,e*), myometrium and endometrium of the uterus, endocrine and exocrine cells of the pancreas, spleen, liver and thymus. Specific areas of TIMP-1 expression were seen in the placenta (Fig. 11.5*f,g*), in the parafollicular cells of the thyroid (Fig. 11.5*h,i*) and in the glandular cells of the prostate.

11.5 Discussion

This is the first study to localise the expression of TIMP-1 mRNA in whole primate ovaries. In common with previously studied species, such as the human (Duncan *et al.*, 1996c) and the ruminant (Smith GW *et al.*, 1994), the marmoset corpus luteum expresses large amounts of TIMP-1 message. As TIMP-1 protein

	Localisation of TIMP-1 message				
	None	Thecal	Granulosal	Both	Interface
Pre-Antral	4	19	0	0	1
Antral	0	47	1	2	1
Small	0	43	2	2	1
Medium	0	33	1	6	1
Large	0	0	9	2	0
Atretic	1	0	0	2	31

Table 11.1

Localisation of TIMP-1 mRNA in marmoset follicles

Localisation of TIMP-1 message in marmoset follicles after *in situ* hybridisation. The grain distribution over the thecal and granulosal layers of each follicle >200 μm was recorded. The numbers are the number of follicles with grains over the thecal layer only, the granulosal layer only, no specific grains, grains over both layers and grains over both layers with particular localisation to the granulosal-thecal interface. Follicles were classified as atretic or normal and normal follicles were classified by size as pre-antral/antral (<600 μm), small (<1 mm), medium (<1.5 mm) or large (≥1.5 mm). The shaded box is the modal category for each follicle class.

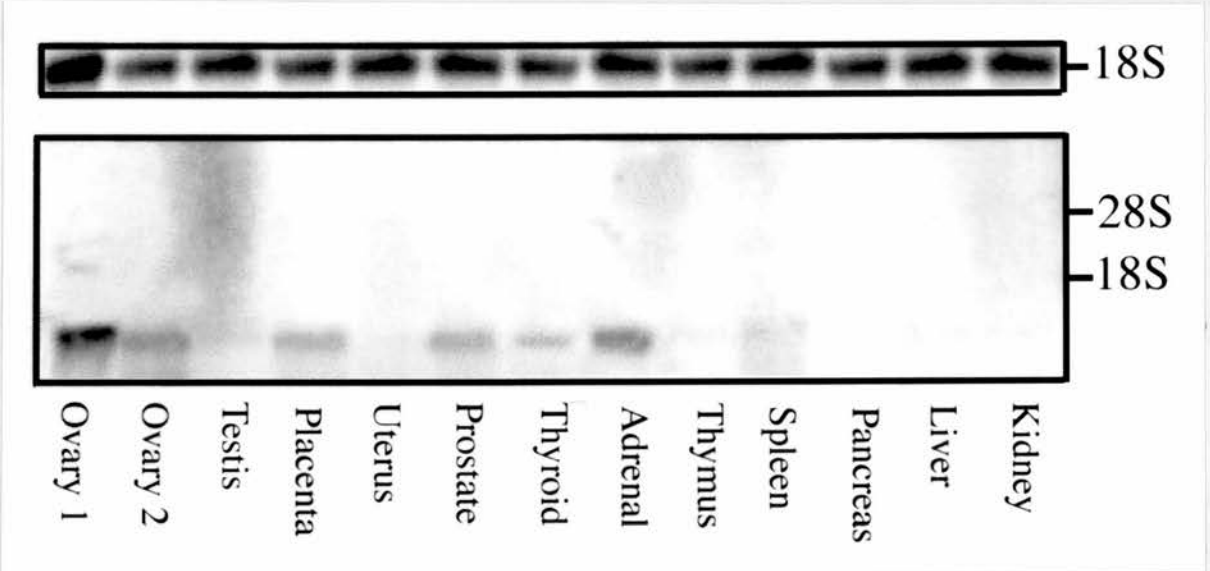


Figure 11.4

Expression of TIMP-1 in different marmoset tissues

Northern blot of TIMP-1 mRNA in different organs from the marmoset. The positions of the ribosomal 28S and 18S bands are indicated. The 18S bands are shown to demonstrate equal RNA loading between lanes. Ovary 1 was from the luteal phase and ovary 2 was from the follicular phase of the ovarian cycle.

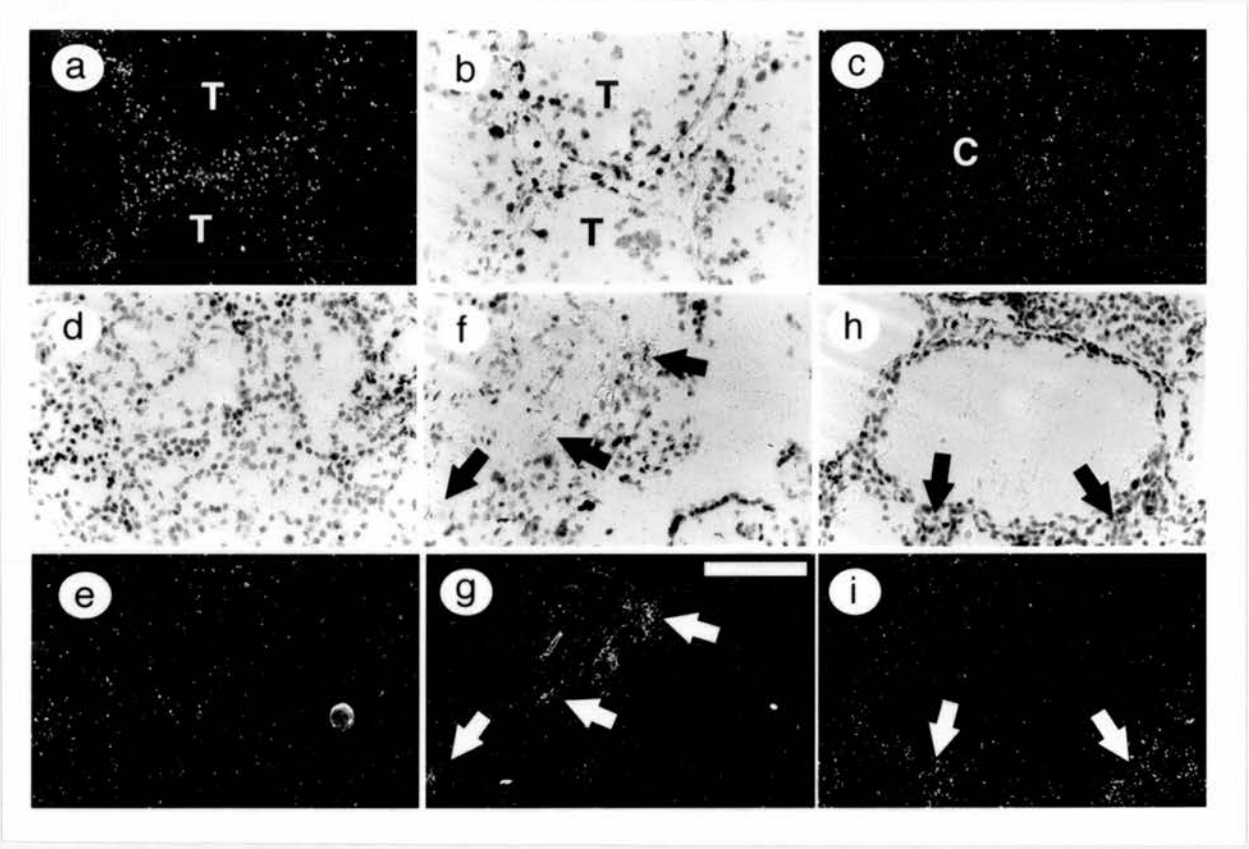


Figure 11.5

Localisation of TIMP-1 mRNA in different marmoset tissues

In situ hybridisation for TIMP-1 mRNA in various primate organs: **a)** dark-field of marmoset testis after *in situ* hybridisation for TIMP-1 with grains seen at the outside of the tubules (T); **b)** light-field of (a) showing the tubules (T) and the basal Sertoli cells; **c)** dark field of the marmoset adrenal gland after TIMP-1 *in situ* hybridisation showing a general increased grain distribution over the cortex (C); **d)** light-field of the marmoset kidney; **e)** dark-field of section (d) showing few grains over the tissue; **f)** light-field of the placenta with cells at the periphery of the trophoblast layer highlighted (arrows); **g)** dark-field of section (f) showing TIMP-1 to be localised to specific areas of the section (arrows); **h)** light-field of the marmoset thyroid gland with the some of the parafollicular cells highlighted (arrows); **i)** dark-field of section (h) showing TIMP-1 to localise to these parafollicular cells (arrows) by *in situ* hybridisation. (Scale bar = 100 μm).

synthesis has been demonstrated in these species (Smith *et al.*, 1993; Jeungel *et al.*, 1994; Duncan *et al.*, 1996c), it is likely that TIMP-1 is also a major secretory product of the marmoset corpus luteum.

Induction of luteolysis resulted in a marked fall in the level of TIMP-1 expression in the marmoset corpus luteum. The mechanism and control of this fall, and its role in tissue remodelling is unclear. The fall in TIMP-1 expression may facilitate increased metalloproteinase digestion of the luteal matrix during regression. However, although rat and bovine corpora lutea express metalloproteinases (Endo *et al.*, 1993a; Tsang *et al.*, 1995), and TIMP-2 is also expressed in ovine corpora lutea (Smith *et al.*, 1995a), detailed information about the expression of these factors, their localisation, and relationship to TIMP-1 during induced luteolysis is not yet available.

The timing of the fall in TIMP-1 expression in association with the drop in progesterone concentration would also be consistent with a recently proposed steroidogenic role for TIMP-1 (Boujrad *et al.*, 1995). There is, however, no indication from these data as to whether the observed changes in TIMP-1 expression are a cause or a consequence of the reduced steroid synthesis. A further consideration is that significant changes in luteal morphology can be seen by 24 hours after induced luteal regression in the marmoset (Fraser *et al.*, 1995b). The fall in TIMP-1 expression may therefore be related to the death of viable luteal cells in the corpus luteum at this time (Fraser *et al.*, 1995c; Young *et al.*, 1997).

These findings are contrary to previous observations of the effects of induced luteolysis in the cow (Juengel *et al.*, 1994). TIMP-1 expression in the bovine corpus luteum was found to increase up to 24 hours post-$PGF_{2\alpha}$ injection and then return to pre-treatment levels within 48 hours. This occurs at a time when increasing cell death is observed in the bovine corpus luteum (Jeungel *et al.*, 1993). We have examined TIMP-1 expression at 12 and 24 hours after induced luteolysis, and found a marked and persistent fall in TIMP-1 expression. However, the luteolytic processes of the primate and the ruminant are different in both nature and time-span (Auletta and Flint, 1988) and this study provides further evidence of these differences.

We have shown that TIMP-1 is also expressed by follicles in the primate ovary and that the localisation changes relative to the apparent size of the follicle. These

data are limited in that two-dimensional interpretation of three-dimensional pre-ovulatory follicles may lead to an underestimation of the size and nature of some follicles. Volume changes may also result from processing and sectioning of the tissue. In spite of these deficiencies, a consistent pattern was observed in the follicular localisation of TIMP-1 mRNA relative to the size of the follicle.

TIMP-1 is first expressed in the thecal cell layer of preantral follicles which are larger than 200 μm in diameter. This is the stage when the follicle becomes gonadotrophin-dependent (Zeleznik and Fairchild Benyo, 1994), and LH receptors can first be detected in the theca (Richards and Midgley, 1976). In the rat, LH stimulates the secretion of a TIMP-like protein and increases TIMP-1 mRNA (Mann *et al.*, 1991; Morgan *et al.*, 1994). It is therefore possible that TIMP-1 expression in the thecal cells is related to the action of LH. In the testis, TIMP-1 production by the Sertoli cells is induced by FSH (Ulisse *et al.*, 1994, Boujrad *et al.*, 1995). Although the granulosa cells express FSH receptors at this stage, and respond to FSH (Zeleznik and Fairchild Benyo, 1994), they do not express TIMP-1 mRNA. It is therefore likely that the control of TIMP-1 expression by FSH is different in the ovarian follicle and the testicular tubule.

In contrast, all the large pre-ovulatory follicles (≥1.5 mm) expressed TIMP-1 in the granulosa cells. Smith GW *et al.* (1994) showed that the ovulatory gonadotrophin surge induced granulosa cell TIMP-1 expression in the sheep. Our results are consistent with a similar preovulatory induction of TIMP-1 in the granulosa cells of the primate ovary. As the granulosa cells express the LH receptor at this stage (Richards and Midgley, 1976), the increased TIMP-1 expression may be directly induced by LH. It is not clear why the theca cells stop expressing TIMP-1, but this pattern of TIMP-1 expression is seen in the granulosa-derived and the theca-derived cells of the human corpus luteum throughout its functional life-span (Duncan *et al.*, 1996c).

The expression of TIMP-1 in the atretic follicles was very different. This was independent of luteolytic treatment or stage of the ovarian cycle. There was a patchy expression in the thecal and granulosal cell layers but marked expression around the basal lamina at the cellular interface. During follicular atresia, the steroidogenic cells degenerate (Hay *et al.*, 1976) and the basal lamina breaks down (Bagavandoss *et al.*, 1983). These data are consistent with a role for MMP involvement in the tissue remodelling associated with follicular atresia. The expression of TIMP-1 may be induced to control local metalloproteinases

involved in the breakdown of the basal lamina. However, the expression of metalloproteinases in the atretic follicle has not yet been described, but they have been shown to be expressed during the breakdown of the follicle wall during ovulation (Reich *et al.*, 1985; Russell *et al.*, 1995).

The physiological role of TIMP-1 in the steroidogenic cells of the follicle is not clear. It is likely that it is involved in the regulation of the matrix remodelling which occurs during follicular growth and development. TIMP-1 has been shown to have proliferative effects on cells *in vitro* (Hayakawa *et al.*, 1992), but it remains to be established whether it can function as a growth factor *in vivo*. In addition, the pattern of TIMP-1 expression is similar to the localisation of several steroidogenic enzymes in different sizes of follicle (Richards *et al.*, 1995). This is consistent with a role for TIMP-1 as a steroidogenic agent.

TIMP-1 was also found to be expressed in other tissues. This is not surprising as TIMP-1 is involved in tissue remodelling throughout the body (Salamonsen, 1996). The luteal phase ovary expressed far higher levels of TIMP-1 mRNA than any of the other tissues investigated. Expression in the placenta and adrenal has previously been noted in the ewe (Hampton *et al.*, 1995). TIMP-1 expression has previously been reported in human endometrium (Hampton and Salamonsen, 1994), but we did not detect it in the marmoset endometrium. This may be because the marmoset monkey does not have a menstrual cycle and lacks cyclical endometrial remodelling. Sertoli cell expression of TIMP-1 has been reported in the rat (Boujrad *et al.*, 1995). As the vast majority of total testicular RNA is of germ cell origin, we were best able to confirm its expression in the marmoset testis by *in situ* hybridisation.

TIMP-1 was particularly expressed by steroidogenic tissues such as the ovary, placenta, testis and adrenal. This expression was generally localised to the steroidogenic cells of these organs. Although the ovary and placenta undergo extensive remodelling during their life-span, the adult adrenal and testis do not. Whether TIMP-1 has any role in these tissues other than specific inhibition of metalloproteinases has yet to be determined. However, these data are consistent with an additional role for TIMP-1 as a facilitator of steroidogenesis *in vivo*. The ontogeny and the role of TIMP-1 in endocrine glands requires further investigation and functional studies of TIMP-1 are required.

In conclusion, the fall in TIMP-1 expression in association with primate luteolysis, and its changing localisation during the life-span of the follicle support a role for modulation of TIMP-1 in the control of tissue remodelling. In addition, these data and the predominant expression of TIMP-1 in steroidogenic tissues, support the concept that TIMP-1 has an additional role as a facilitator of role in steroidogenesis.

Chapter 12

The effect of luteal 'rescue' on the expression and localisation of MMPs and TIMPs in the human corpus luteum

12.1 Abstract

Luteolysis is associated with tissue remodelling likely to involve the MMPs and their specific tissue inhibitors, the TIMPs. This study investigated the expression and localisation of the major MMPs and TIMPs in the human corpus luteum throughout the luteal phase and after luteal 'rescue' with hCG. Corpora lutea (n=9) were collected at hysterectomy and dated by serial urinary LH estimation. In addition, corpora lutea (n=3) were collected from women who had received daily doubling doses of hCG to mimic the hormonal changes of early pregnancy. MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-3 were investigated by zymography, reverse zymography, northern blotting and *in situ* hybridisation. There was no change in the expression of MMP-1, TIMP-1 and TIMP-2 throughout the luteal phase or after luteal 'rescue'. Little TIMP-3 could be detected in the corpus luteum. MMP-9 activity peaked in the early and late-luteal phase. The expression and activity of MMP-2 was maximal in the late-luteal phase. Exposure to hCG during luteal 'rescue' *in vivo* was associated with a reduction ($p<0.05$) in the expression and activity of MMP-2. Messenger RNA for MMP-1, MMP-2 and TIMP-2 were localised to the connective tissue stroma and the theca-lutein cells of the corpus luteum. In contrast, TIMP-1 mRNA was localised to the granulosa-lutein cells and MMP-9 mRNA was expressed in scattered cells within the steroidogenic and non-steroidogenic cell layers. In conclusion, during maternal recognition of pregnancy, hCG prevents the normal increase in MMP-2 in the late-luteal phase. MMPs can function in an environment containing large amounts of TIMP-1 as they have a different cellular localisation.

12.2 Introduction

Unless hCG is secreted from the implanting blastocyst, the human corpus luteum will undergo structural and functional luteolysis (Behrman *et al.*, 1993). The corpus luteum changes from the most active endocrine gland in the body, with a blood flow per unit mass much greater than the kidney (Ford *et al.*, 1982), to a small fibrous remnant in a matter of days. This extensive tissue remodelling is likely to involve a group of zinc-dependent proteolytic enzymes known as the MMPs (Luck and Zhao, 1995; Salamonsen, 1996; Hulboy *et al.*, 1997). These enzymes have been implicated in a wide variety of biological processes which involve remodelling of the ECM such as ovulation, menstruation, angiogenesis, and tumour growth and metastasis (Reich *et al.*, 1985; Hampton and Salamonsen, 1994; Naylor *et al.*, 1994).

The activity of MMPs is controlled at several levels, including synthesis as pro-enzymes, enzyme activation, and the production of specific tissue inhibitors (Matrisian, 1990; Birkedal-Hansen, 1995). TIMPs are of particular interest as TIMP-1 is one of the major products of the corpus luteum. It is produced in large amounts by the corpus luteum of many species, including the rat (Nothnick *et al.*, 1995), sheep (Smith GW *et al.*, 1994), cow (Juengel *et al.*, 1994), pig (Smith MF *et al.*, 1994), monkey, as shown in **Chapter 11**, (Duncan *et al.*, 1996b) and the human, as shown in **Chapter 10**, (Duncan *et al.*, 1996c). In addition, it has recently been reported that TIMP-2 is also produced by corpora lutea of rats (Nothnick *et al.*, 1995), sheep (Smith *et al.*, 1995a) and cows (Smith *et al.*, 1996b), and that TIMP-3 can also be detected in rat ovaries (Nothnick *et al.*, 1995).

TIMPs bind to and inhibit MMP enzymes with a one-to-one stoichiometry (Birkedal-Hansen, 1995). As TIMP-1, in particular, is produced in large amounts throughout the normal luteal phase (Duncan *et al.*, 1996c), it is not clear how metalloproteinase enzymes function in an environment containing large amounts of specific inhibitor. This study aimed to investigate the expression and localisation of the common MMPs and TIMPs in the human corpus luteum throughout the normal luteal phase and the effect of luteal 'rescue' with exogenous hCG, to mimic the hormonal changes of early pregnancy, on these enzymes and their inhibitors.

12.3 Specific Materials and Methods

12.3.1 Tissues Studied

Corpora lutea were enucleated at the time of hysterectomy in women undergoing surgery for benign conditions (**2.2**). On the basis of urinary LH estimation (**2.2.4**), three corpora lutea classified as early-luteal, three as mid-luteal and three as late-luteal were investigated. Three corpora lutea obtained after luteal 'rescue' with hCG (**2.2.2**) were also studied. A further corpus luteum was obtained (**2.2.3**) from a woman who had received hCG for 8 days to achieve luteal 'rescue' (**2.2.2**) but the operation was postponed. This corpus luteum was collected 3 days after the final hCG injection.

Two pieces of each corpora lutea were stored at -70 °C for subsequent protein and RNA extraction (**2.2.3**). One piece was frozen in embedding medium and stored at -70 °C (**2.2.3**). Serial frozen sections (6 μm) were cut onto RNAse-free slides coated with poly-L-lysine (**3.2.1**) and stored at -70 °C until use. In each case, an endometrial biopsy was fixed in 4% paraformaldehyde and processed into paraffin wax for luteal phase-dating (**3.2.8**). Fresh human placental tissue was obtained from the local maternity hospital.

12.3.2 Gelatine Zymography

Protein was extracted from corpora lutea in 0.1% (w/v) SDS at 4 °C. The protein content of the sample after sonication was measured using the method of Bradford (1976). Seventy-five micrograms of protein in sample buffer [10% (v/v) glycerol, 1% (w/v) SDS and 0.04% (v/v) bromophenol blue] were applied, without heating or reduction, to an 11% (w/v) polyacrylamide gel containing 1 mg/ml gelatine and 0.1% (w/v) SDS. After electrophoretic separation of proteins, the gels were incubated in 2.5% triton X-100 for 30 min to remove the SDS. The gels were then incubated for 16 hours at 37 °C in 50 mM Tris-HCl (pH 7.6), containing 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% (w/v) Brij 35. The gels were stained in staining solution [30% (v/v) methanol, 10% glacial acetic acid, 0.5% (w/v) Coomassie brilliant blue G250] and then destained in the same solution in the absence of dye.

12.3.3 Reverse Zymography

Reverse zymography using 75 μg of each protein sample was performed using a commercial kit (**3.1.1**). Briefly, 12% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS, 1 mg/ml gelatine and a solution of secreted MMPs (as supplied) were prepared. After electrophoresis, the gels were washed overnight in a solution of 2.5% Triton X-100, 50 mM Tris-HCl (pH 7.5) and 5 mM $CaCl_2$. The gels were rinsed in water and incubated in 50 mM Tris-HCl (pH 7.5) and 5 mM $CaCl_2$, with gentle shaking, for 24 h at 37 °C. Staining and destaining were carried out as described above (**12.3.3**), and bands corresponding to TIMP-1, TIMP-2 and TIMP-3 were identified by reference to the standards supplied with the kit (**3.1.1**).

12.3.4 Northern Blotting

Total RNA (20 μg) was denatured, electrophoresed in a 1.5% formaldehyde-agarose gel, transferred to a nylon membrane and fixed by u.v. cross-linkage (**3.6.1**). Northern blot analysis was conducted using ^{32}P dCTP-labelled cDNA probes (**3.5.5**) for MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-3 (**3.1.3**) as described previously (**3.6.2**). After washing (**3.6.3**), the blots were laid down to a phosphor screen for 48-72 h and visualised using a phosphorimager computer (**3.6.3**). The blots were then stripped (**3.6.4**) and then reprobed with a ^{32}P end-labelled oligonucleotide which hybridises to 18S RNA (**3.6.4**). The molecular size of the bands was calculated with reference to standard RNA molecular weight markers run in an adjacent lane (**3.6.1**).

12.3.5 *In situ* Hybridisation

Isotopic *in situ* hybridisation was performed on frozen sections using ^{35}S-labelled riboprobes (**3.7**). Antisense and sense riboprobes incorporating ^{35}S-labelled UTP were prepared (**3.7.2**) from the appropriate plasmids (**3..1.3**). The riboprobes were generated from the above plasmids (**3.7.1**) using the following restriction enzymes (**3.5.3**) and RNA polymerases (**3.7.2**): MMP-1, HindIII with T7 polymerase (antisense) and NotI with T3 polymerase (sense); MMP-2, EcoR1 with T7 polymerase (antisense) and HindIII with SP6 polymerase (sense); MMP-9, EcoR1 with T7 polymerase (antisense) and Pst1 with SP6 polymerase (sense); TIMP-1, Kpn1 with T7 polymerase (antisense) and HindIII with SP6 polymerase (sense); TIMP-2, HindIII with SP6 polymerase (antisense) and EcoR1 with T7 polymerase (sense).

The sections were prepared as described previously (**3.7.3**) and hybridisation was conducted at 55 °C using 1 x 10^6 c.p.m ^{35}S-labelled antisense riboprobe (**3.7.4**). The ^{35}S-labelled sense riboprobe (1 x 10^6 c.p.m.) (**3.7.2**) was added to serial sections as a negative control (**3.7.4**). After washing in increasingly stringent conditions (**3.7.5**), the slides were dipped in photographic emulsion (**3.7.6**) and incubated at 4 °C for 21 days in the dark. After developing and fixing (**3.7.6**), the sections were washed, counterstained with haematoxylin and mounted (**3.2.8**).

12.3.6 Immunohistochemistry

Frozen sections on poly-L-lysine-coated slides (**3.2.1**) were prepared as described previously (**3.2.3**). Non-specific binding was blocked using NGS with 5% (w/v) BSA. The primary antibody to 17α-hydroxylase (**3.1.2**) was diluted to a concentration of 1:1500 in TBS and applied to the section for 20 hours at 4 °C (**3.2.6**). Antibody binding was visualised with an AB-AP complex using biotinylated goat anti-rabbit immunoglobulins as the secondary antibody (**3.2.6**). Colouration was achieved using a substrate to give a red end-product (**3.2.7**). Sections were then counterstained with haematoxylin, dehydrated, and mounted (**3.2.8**).

12.3.7 Analysis of Results

The intensities of the 92 kDa and 66 kDa bands detected by zymography were measured by computer-aided densitometric image analysis (NIH Image 1.55) after image capture and inversion. Northern blot band intensity was measured using the phosphorimager computer. To correct for minor differences in loading, the ratio of the band intensity relative to the 18S band was used for data analysis. One-way ANOVA was used to investigate differences in expression throughout the luteal phase. The 'rescued' corpora lutea were compared to the late-luteal corpora lutea using an unpaired t-test. A commercial software package was used for statistical analysis (StatView 4.0).

12.4 Results

12.4.1 Plasma Progesterone Concentrations

The classification of the corpora lutea by serial urinary LH measurement agreed with the luteal-phase dating of endometrial biopsies using the method of Li *et al.* (1988). The plasma progesterone concentrations were 35.3 ±9.8 nmol/l in the early-luteal samples, 41.0 ±9.9 nmol/l in the mid-luteal samples and 19.2 ±12.9 nmol/l in the late-luteal samples. After luteal 'rescue' by exogenous hCG the plasma progesterone concentrations had increased to 52.6 ±1.5 nmol/l. The plasma progesterone concentration in the post-'rescue' sample was 9.16 nmol/l.

12.4.2 Identification of Metalloproteinases and their Tissue Inhibitors

Three distinct bands of gelatinase activity at 92 kDa, 72 kDa and 66 kDa were detected in the human corpus luteum by gelatine zymography (Fig. 12.1). These are consistent with MMP-9, and the latent and active form of MMP-2 respectively (Endo *et al.*, 1993a; Salamonsen, 1996). Reverse zymography demonstrated a band of inhibition of gelatinase activity at approximately 28 kDa and a lighter band at 21 kDa (Fig. 12.2). These correspond to TIMP-1 and TIMP-2 respectively (Hampton *et al.*, 1995; Salamonsen, 1996). A further band at 24 kDa was seen in human placental tissue, but was absent from corpora lutea. This is consistent with TIMP-3 (Hampton *et al.*, 1995) which is produced by decidual tissue (Higuchi *et al.*, 1995). TIMP-1 and TIMP-2 could be detected in samples taken from different stages of the luteal phase and after luteal 'rescue' with exogenous hCG (Fig. 12.2).

The activities of MMP-2 and MMP-9 changed over the luteal phase (Fig. 12.3). MMP-9 activity peaked in the early- and late-luteal phase and was lowest in the mid-luteal phase ($p<0.05$), In contrast, MMP-2 activity increased throughout the luteal phase to a maximum in the late-luteal phase ($p<0.05$). Luteal 'rescue' with hCG resulted in lower MMP-2 activity than during the late-luteal phase in the absence of hCG ($p<0.05$). When the corpus luteum was 'rescued' with hCG, and then the trophic support was withdrawn (in the post-'rescue' sample), large amounts of MMP-2 activity was clearly identified by zymography (Fig. 12.1).

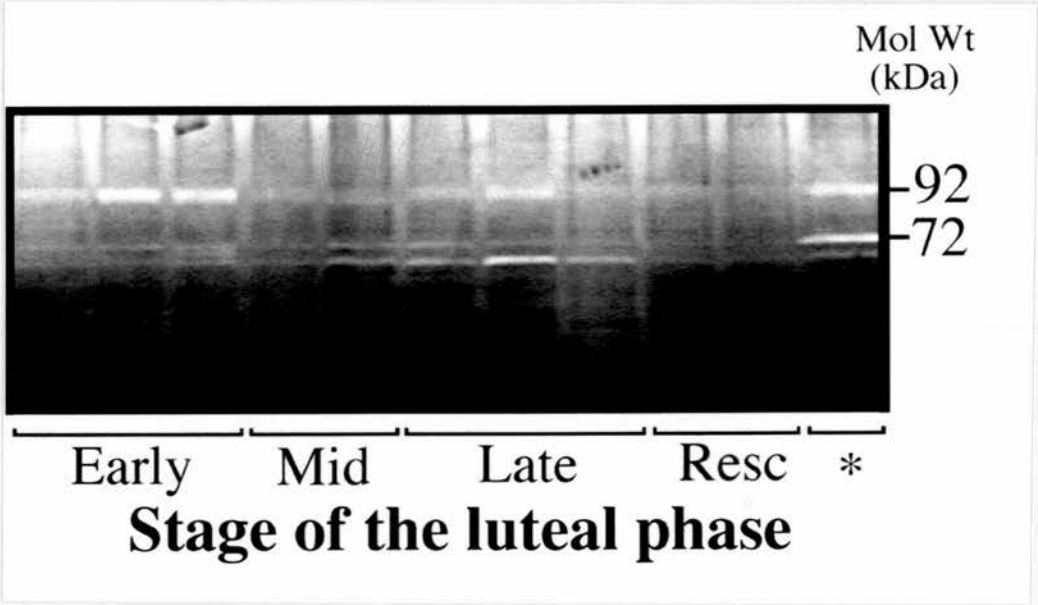


Figure 12.1

Gelatine zymogram from human corpora lutea

Representative gelatine zymogram of human corpora lutea extracts from the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late- (LH+11 to LH+14) luteal phase and after luteal 'rescue' by hCG (hCGx6 to hCGx8). The extract marked (*) is taken from a corpus luteum which was 'rescued' with hCG for 8 days and then collected 3 days after the final exposure to hCG. The bands are bright against a dark background and the molecular size of each band in kDa is indicated on the right.

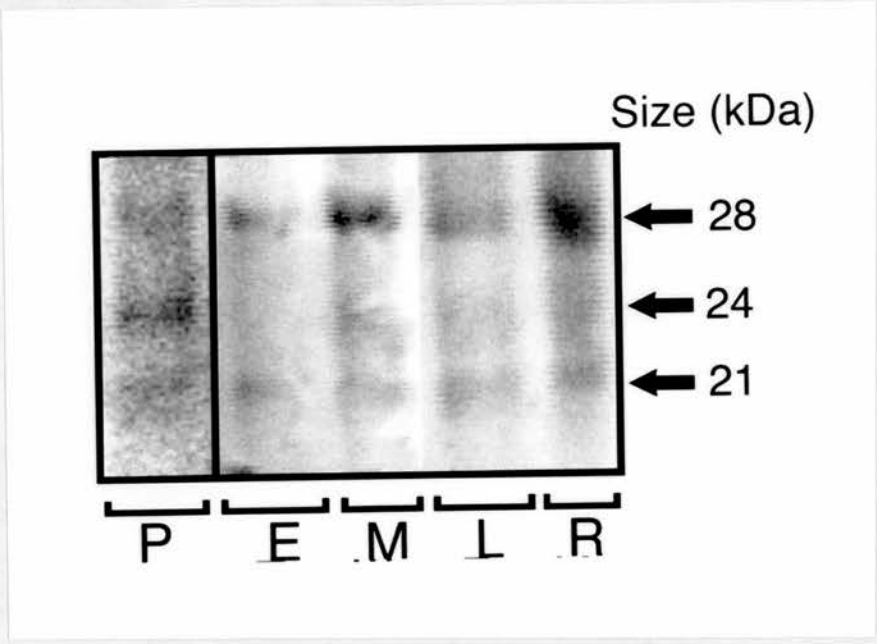


Figure 12.2

Reverse zymogram from human corpora lutea

Representative reverse zymogram of protein extracts from human placenta (P) and corpora lutea collected in the early- (E) (LH+1 to LH+5), mid- (M) (LH+6 to LH+10) and late-luteal (L) (LH+11 to LH+14) phase, and after luteal 'rescue' (R) with exogenous hCG (hCGx6 to hCGx8) *in vivo*. The bands are seen as dark against a lighter background and the molecular size of each band in kDa is indicated on the right.

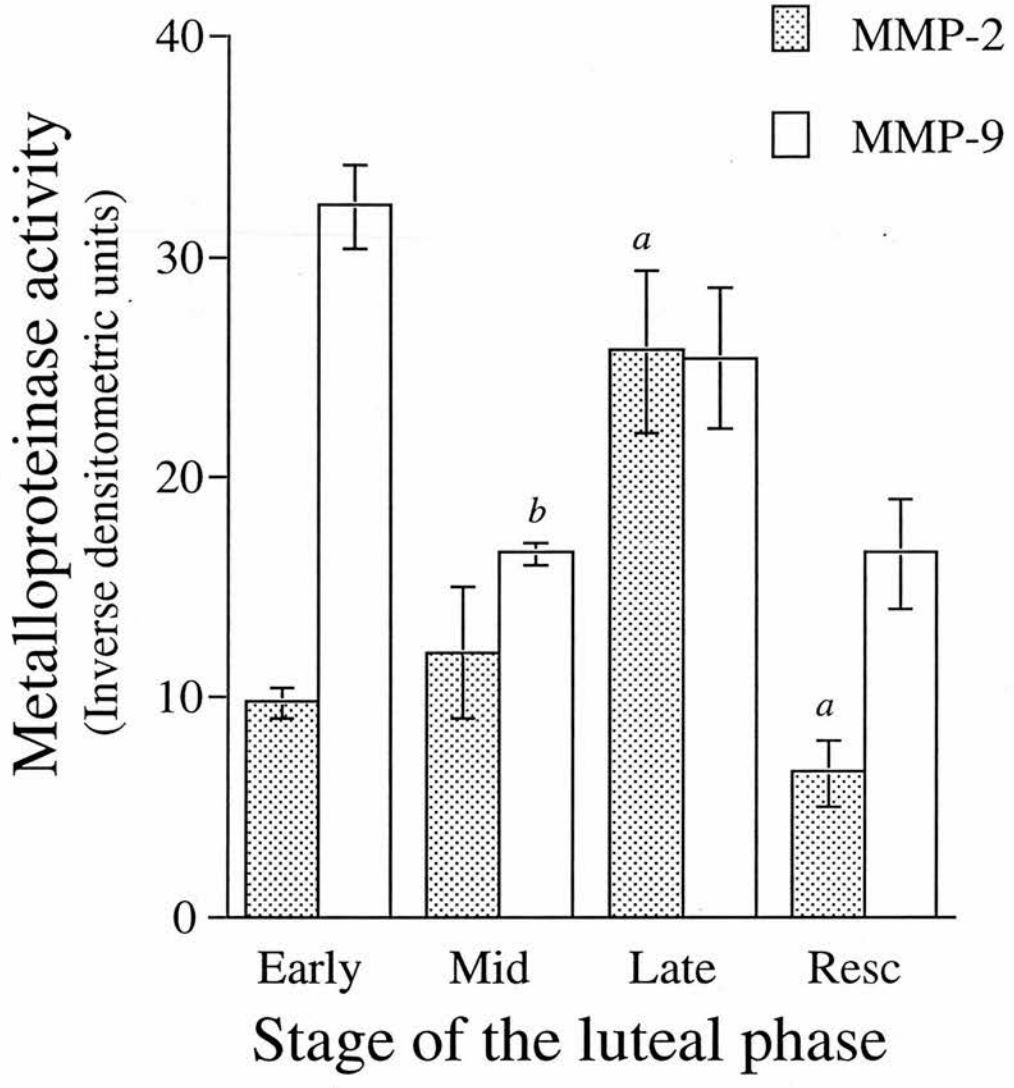


Figure 12.3

Activities of MMP-2 and MMP-9 in human corpora lutea

Activities of MMP-2 and MMP-9 in human corpora lutea. The inverse intensity of the bands for MMP-9 (92 kDa) and the active form of MMP-2 (66 kDa) on gelatine zymography in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late-luteal (LH+11 to LH+14) phase and after luteal 'rescue' with hCG (hCGx6 to hCGx8). Values are means ± standard deviation (S.D.) (n=3 per group). Differences ($p<0.05$) in mean activities are shown (*a*: t-test; *b*: ANOVA).

12.4.3 Expression of Metalloproteinases and their Tissue Inhibitors

A single band of approximately 0.9 kb corresponding to TIMP-1 (Rapp *et al.*, 1990; Duncan *et al.*, 1996c) was detected in human corpora lutea by northern blotting (Fig. 12.4). This confirms our previously reported results, in **Chapter 10**, (Duncan *et al.*, 1996c). Northern blotting for TIMP-2 resulted in a single band of 3.6 kb (Fig. 12.4). This is consistent with the transcript size for TIMP-2 mRNA in the human (Stetler-Stevenson *et al.*, 1990). Several mRNA species corresponding to TIMP-3 (Higuchi *et al.*, 1995) were detected in the placenta but were not seen in the human corpus luteum (data not shown). As we have previously reported (Duncan *et al.*, 1996c), there were no significant differences in the level of TIMP-1 expression throughout the luteal phase or after luteal 'rescue' with hCG (Fig. 12.5). Likewise TIMP-2 expression did not appear to change throughout the luteal phase or after luteal 'rescue' (Fig. 12.5).

Specific mRNA transcripts of 3.5 kb were detected in corpora lutea after Northern blotting for MMP-2. This is consistent with the reported transcript size of MMP-2 (Hoeben *et al.*, 1996). In addition, northern blotting for MMP-1 demonstrated transcripts of 3.6 kb and approximately 1.0 kb in human corpora lutea (data not shown). Little MMP-9 expression could be detected by northern blotting in spite of clear identification by zymography. Messenger RNA for MMP-2 was lower ($p<0.05$) in 'rescued' corpora lutea that in the late-luteal phase in the absence of hCG (Fig. 12.6). There were no differences in MMP-1 expression throughout the luteal phase or after luteal 'rescue' with exogenous hCG (Fig. 12.6).

12.4.4 Localisation of Metalloproteinases and their Tissue Inhibitors

Messenger RNA for TIMP-1, TIMP-2, MMP-1, MMP-2 and MMP-9 was localised in human corpora lutea by isotopic *in situ* hybridisation. Each of these mRNA species had a specific pattern of localisation which persisted throughout the normal luteal phase and after luteal 'rescue' with exogenous hCG. In agreement with our previous findings, reported in **Chapter 10**, TIMP-1 was highly expressed in the granulosa-lutein cells of the corpus luteum (Fig. 12.7*a,b*) (Duncan *et al.*, 1996c). In contrast, TIMP-2 was localised to different regions of the corpus luteum. TIMP-2 was expressed at the periphery of the granulosa-lutein cells (Fig. 12.7*c*). Comparison with serial sections immunostained for 17α-hydroxylase, to identify the theca-lutein cells, showed that TIMP-2 was expressed by the theca-

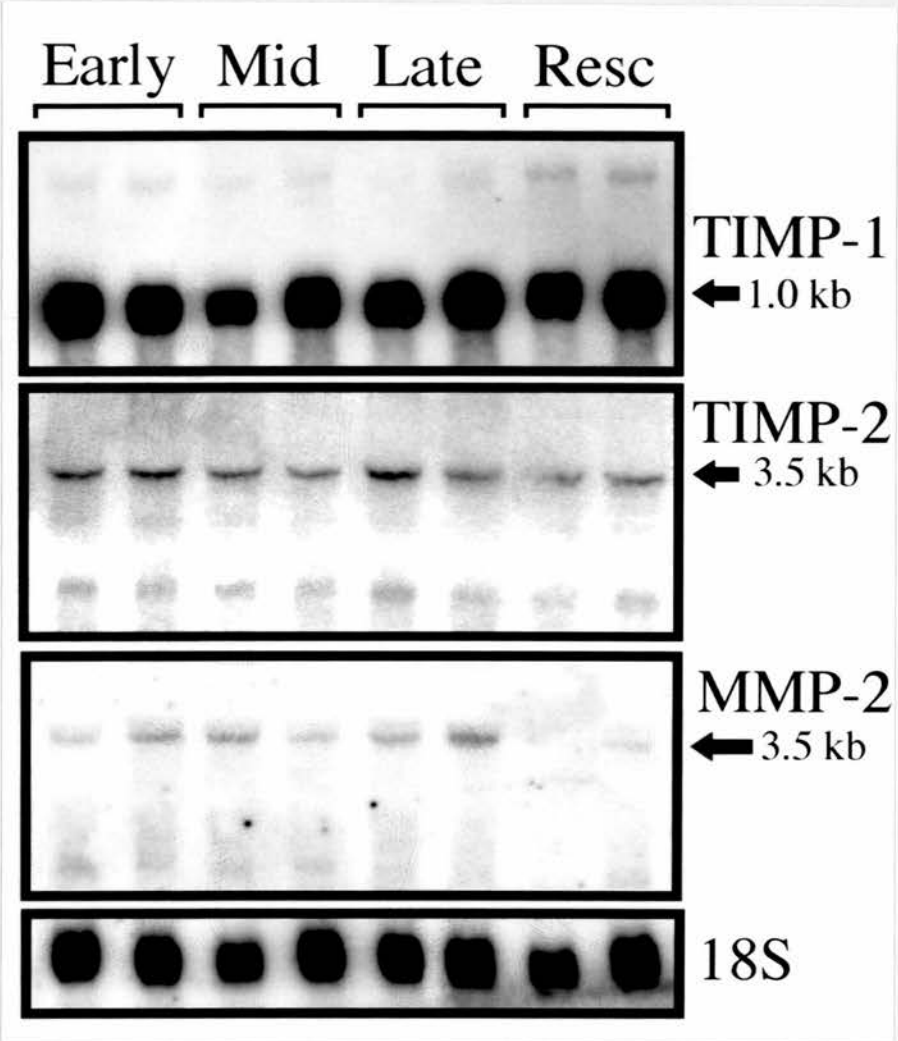


Figure 12.4

Northern blot for TIMP-1, TIMP-2 and MMP-2 in human corpora lutea

Representative northern blot for TIMP-1, TIMP-2 and MMP-2 in human corpora lutea in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late-luteal (LH+11 to LH+14) phase and after luteal 'rescue' with hCG (hCGx6 to hCGx8). Specific hybridisation bands are dark against a lighter background. The approximate size in kb of the bands are indicated and the 18S RNA bands are shown to demonstrate equal RNA loading.

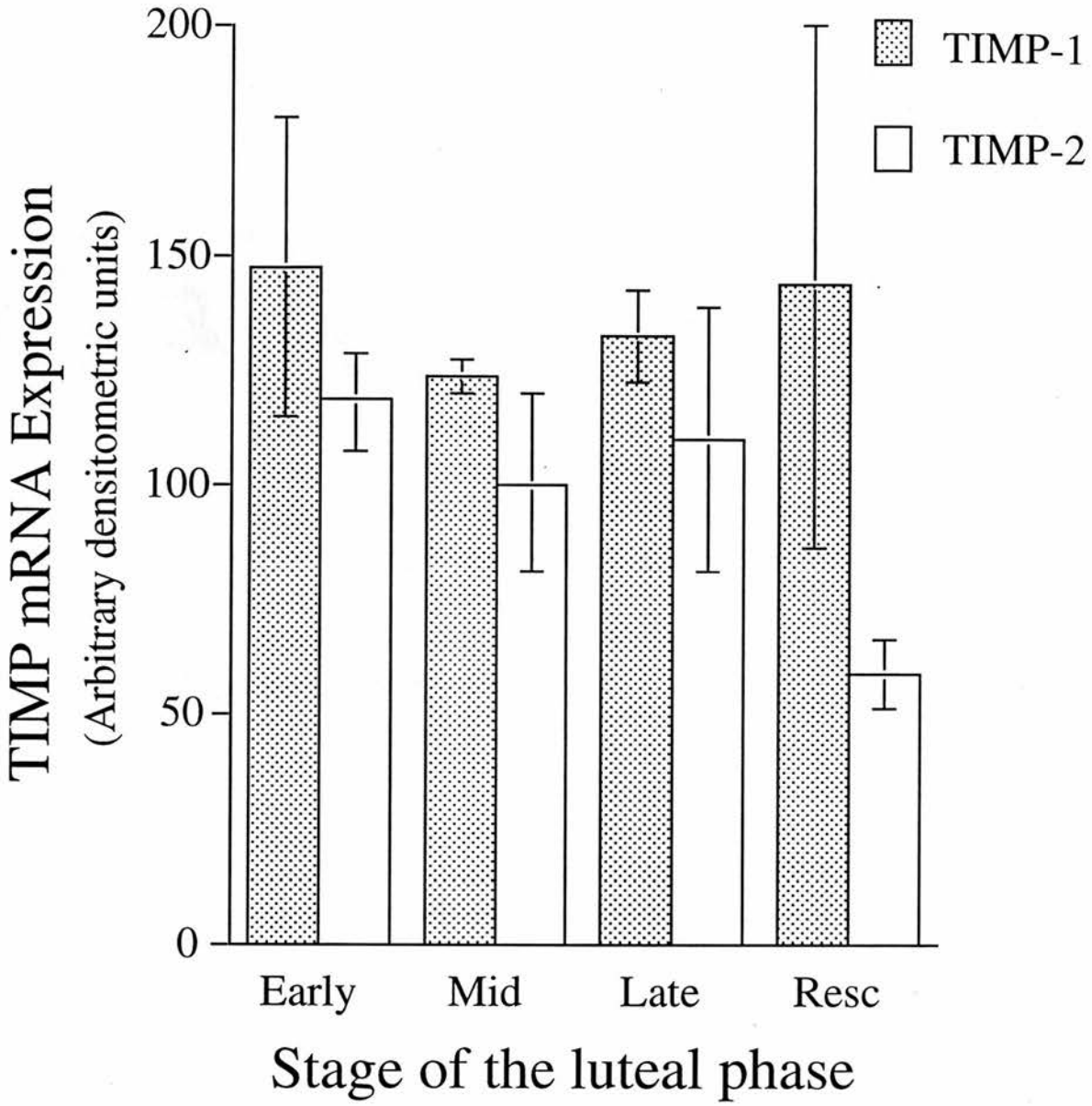


Figure 12.5

Expression of TIMP-1 and TIMP-2 in human corpora lutea

Expression of TIMP-1 and TIMP-2 in the human corpus luteum. The intensity of TIMP-1 and TIMP-2 mRNAs, corrected for 18S intensity, in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late- (LH+11 to LH+14) luteal phase and after luteal 'rescue' with exogenous hCG (hCGx6 to hCGx8) *in vivo*, are shown. Values are means ±S.D. (n=3 per group). There were no significant differences in the level of expression throughout the luteal phase (ANOVA) or after luteal 'rescue' (t-test).

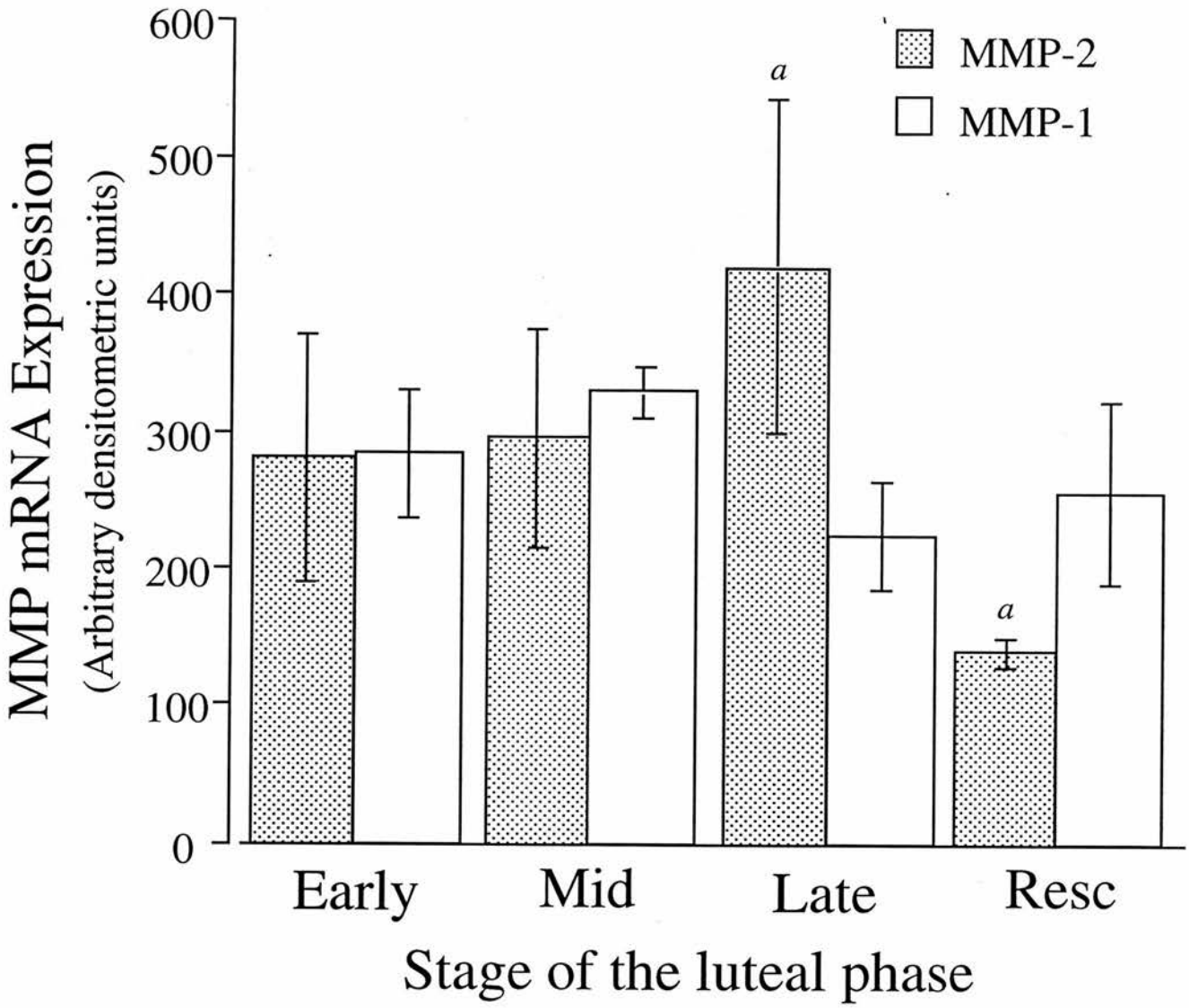


Figure 12.6

Expression of MMP-1 and MMP-2 in human corpora lutea

Expression of MMP-1 and MMP-2 mRNA in the human corpus luteum. The intensities of the major MMP-1 and MMP-2 mRNA bands, corrected for 18S intensity, in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late- (LH+11 to LH+14) luteal phase and after luteal 'rescue' with exogenous hCG (hCGx6 to hCGx8) *in vivo*, are shown. Values are means ±S.D. (n=3 per group). Significant differences are shown (*a*; $p<0.05$) (ANOVA).

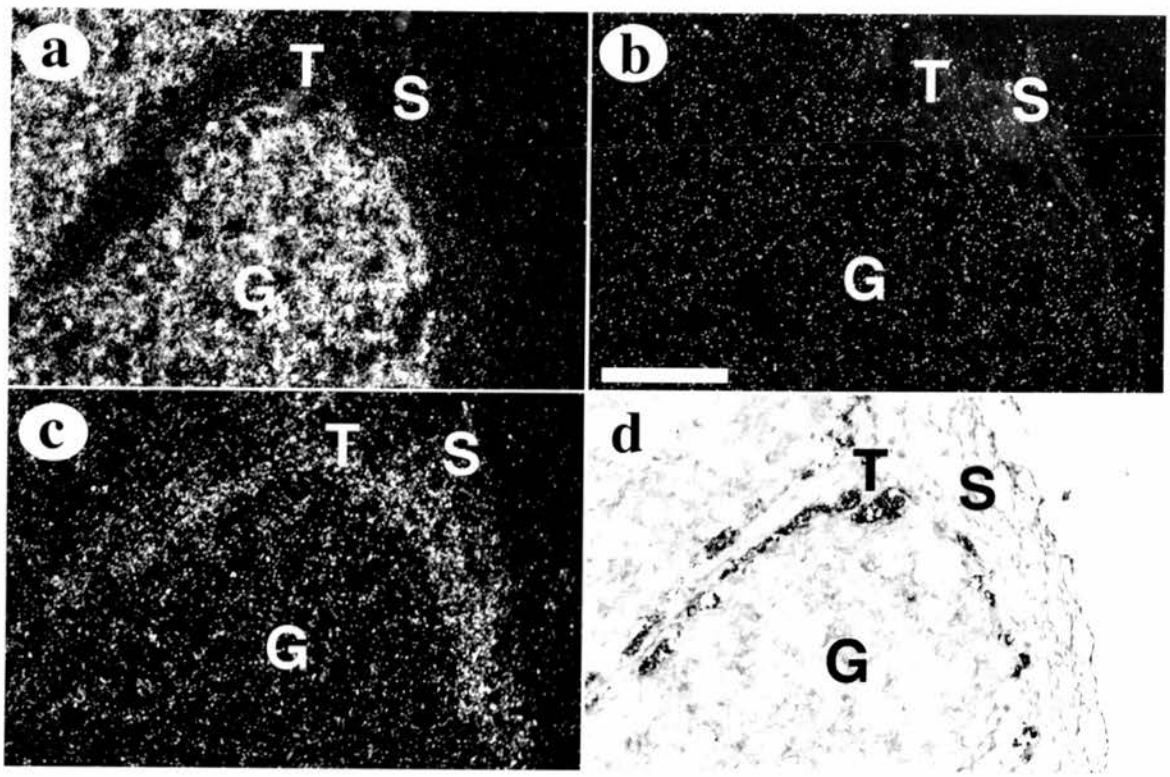


Figure 12.7

Localisation of TIMP-1 and TIMP-2 mRNA in the human corpus luteum

Localisation of TIMP-1 and TIMP-2 mRNA in the human corpus luteum: **a)** dark-field of TIMP-1 *in situ* hybridisation in the early-luteal corpus luteum showing expression in the granulosa-lutein cells; **b)** negative control of (a) showing few silver grains with no specific distribution; **c)** serial section of (a) showing dark-field of TIMP-2 *in situ* hybridisation: expression of TIMP-2 is in a different cellular compartment to TIMP-1; **d)** serial section of (a) immunostained for 17α-hydroxylase to localise the theca-lutein cells. (G)= granulosa-lutein cells; (T)= theca-lutein cells; (S)= connective tissue stroma. Scale bar = 100 μm.

lutein cells (Fig. 12.7*d*). In addition, TIMP-2 was also expressed in the fibrous connective tissue surrounding the steroidogenic cells (Fig. 12.7*c*).

MMP-1 was heavily expressed by the cells of the stroma and connective tissue of the corpus luteum (Fig. 12.8*a*). Only very low levels of expression were seen in the area of the gland containing steroidogenic cells (Fig. 12.8*a,b*). MMP-2 was localised to the theca-lutein cells and surrounding connective tissue (Fig. 12.8*c*). MMP-2 could also be detected around the vasculature, and occasionally in cells within blood vessels. Little MMP-2 expression could be detected in the granulosa-lutein cell population. The distribution of MMP-9 was different, as expression was localised to individual cells within the gland (Fig. 12.8*d*). Although these cells were concentrated along the theca-lutein cell layer and vascular connective tissue, some cells within the granulosa-lutein compartment were also found to express MMP-9 (Fig. 12.8*d*). This pattern of expression of MMPs and TIMPs was consistent, and seen in all serial sections examined (Fig. 12.9).

12.5 Discussion

This paper demonstrates the expression and localisation of the MMPs and their specific tissue inhibitors in the human corpus luteum. TIMP-1 has already been described as a major product of the corpus luteum of several species (Smith GW *et al.*, 1994; Juengel *et al.*, 1994; Smith MF *et al.*, 1994) and we have previously reported its expression in the human (Duncan *et al.*, 1996c) and non-human primate (Duncan *et al.*, 1996b). We have now found that TIMP-2 is also expressed by the human corpus luteum. This agrees with the observation of Smith *et al.* who described TIMP-2 expression in ovine (Smith *et al.*, 1995a) and bovine (Smith *et al.*, 1996b) follicles and corpora lutea. In contrast to TIMP-1 and TIMP-2, TIMP-3 is a not thought to be a secreted molecule but a component of the extracellular matrix (Leco *et al.*, 1994; Salamonsen, 1996). Although we detected TIMP-3 mRNA in the human placenta, where it has previously been described (Higuchi *et al.*, 1995), we found little expression in the corpus luteum. Similarly Uria *et al.* (1994) did not detect TIMP-3 expression in the human ovary. However, TIMP-3 mRNA has recently been reported in the ovary of the pseudo-pregnant rat (Nothnick *et al.*, 1995). As TIMP-3 expression was inversely related to TIMP-1 expression, which decreased in the pseudo-pregnant ovary, this discrepancy may reflect the different time-spans examined, or it may be a species difference.

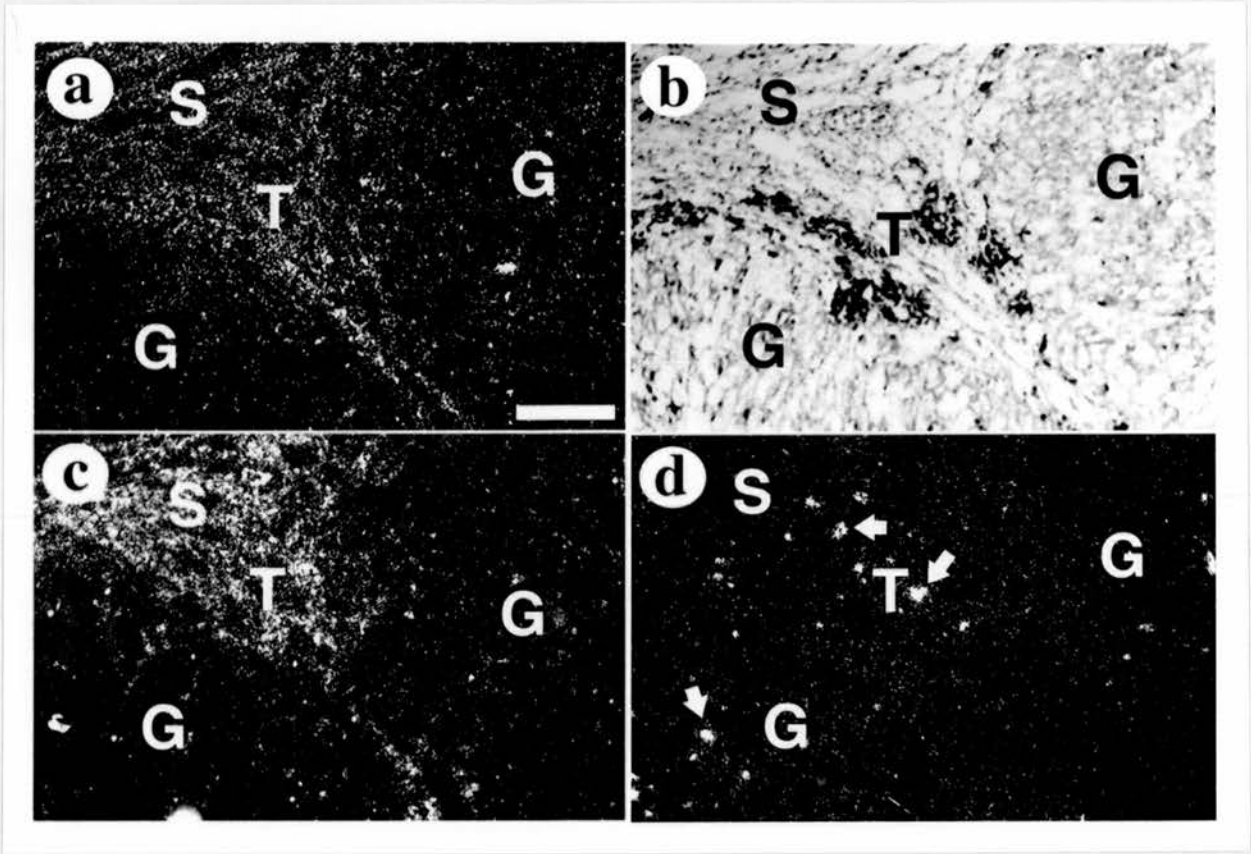


Figure 12.8

Localisation of MMP-1, MMP-2 and MMP-9 mRNA in the human corpus luteum

Localisation of mRNA for the major MMPs in the human corpus luteum: **a)** dark-field of *in situ* hybridisation for MMP-1 in the mid-luteal phase corpus luteum showing expression in the connective tissue stroma with minimal expression in the granulosa-lutein cell layer; **b)** serial section of (a) immunostained for 17α-hydroxylase to localise the theca-lutein cells; **c)** dark-field *in situ* hybridisation showing the localisation of MMP-2 mRNA and **d)** MMP-9 mRNA in the same corpus luteum. (G)= granulosa-lutein cells; (T)= theca-lutein cells; (S)= connective tissue stroma. Scale bar = 100 μm.

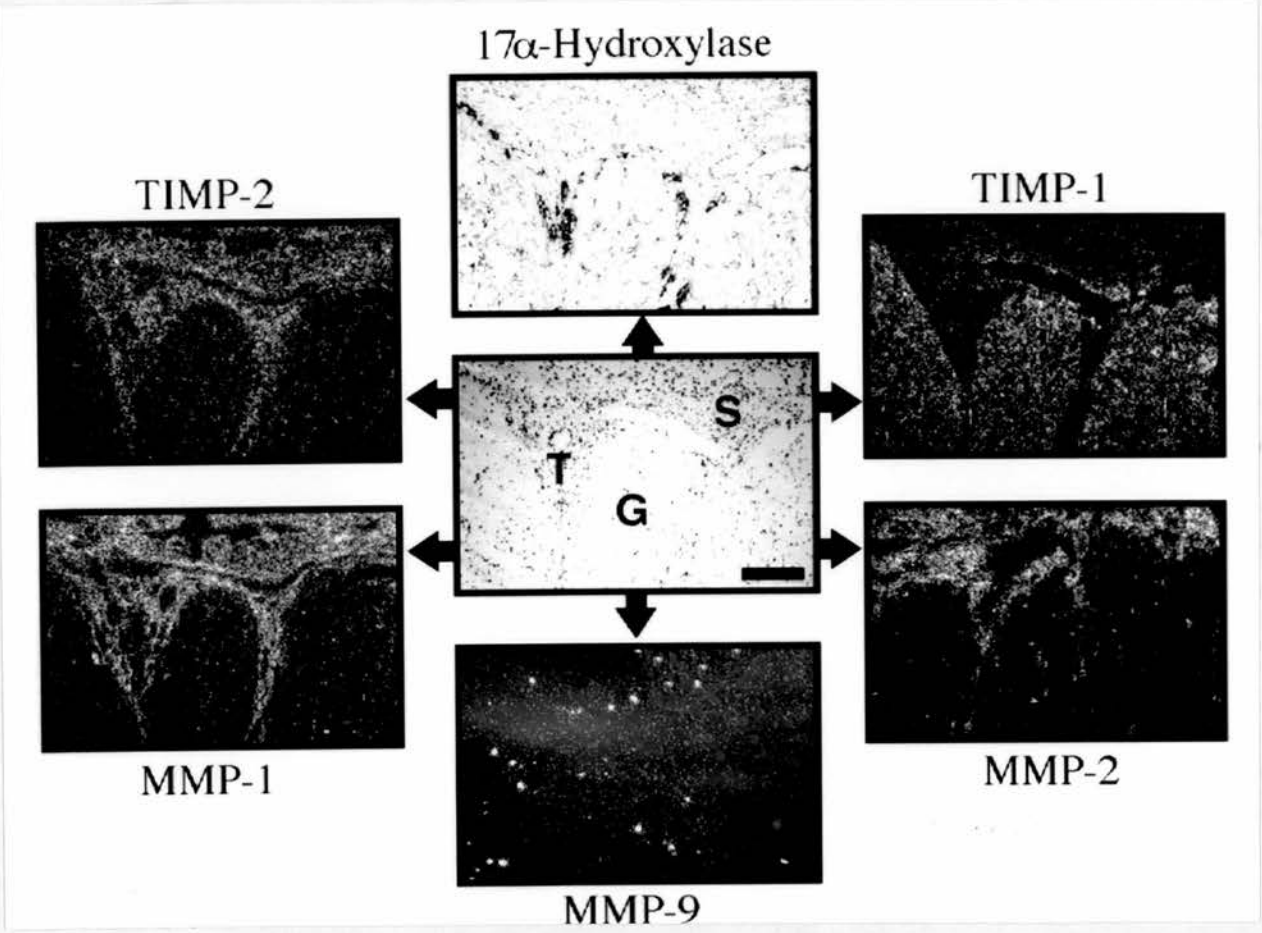


Figure 12.9

Differential localisation of MMPs and TIMPs in the corpus luteum

Relationship between the localisation of the major MMPs and TIMPs in the late-luteal corpus luteum. A composition of serial sections after *in situ* hybridisation for MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2, and immunohistochemistry for 17α-hydroxylase arranged around the light-field section of the corpus luteum showing the pattern of expression of MMPs and TIMPs. (G)= granulosa-lutein cells; (T)= theca-lutein cells; (S)= connective tissue stroma. Scale bar = 200 μm.

Indeed, species differences in luteal TIMP-1 expression during luteolysis have already been described (Juengel *et al.*, 1994; Duncan *et al.*, 1996b; Duncan *et al.*, 1996c). In the human it seems that TIMP-1 and TIMP-2 are the major luteal TIMPs.

In the small numbers we analysed, the expression TIMP-2 did not change during the functional luteal phase or after luteal 'rescue' with exogenous hCG. TIMP-2 expression was found to change during the luteal phase in ovine corpora lutea (Smith *et al.*, 1995a). Smith *et al.* (1995a) reported that TIMP-2 expression was maximal in the early-luteal phase and significantly lower in the late-luteal phase. This is likely to be another example of species difference in the physiology of the corpus luteum. In the cow, TIMP-2 expression was reported to increase significantly from the early- to the mid-luteal phase (Smith *et al.*, 1996b) and expression was increased after prostaglandin-induced luteolysis (Juengel *et al.*, 1994). Further evidence of species difference is that the primary TIMP-2 transcript size in the sheep corpus luteum is 1.0 kb (Smith *et al.*, 1995a), whereas in the human corpus luteum and other tissues (Stetler-Stevenson *et al.*, 1990) the size is 3.5-kb. In the human corpus luteum, control of tissue remodelling during the functional luteal phase does not appear to be related to alterations in the levels of expression of TIMPs.

MMP-1, MMP-2 and MMP-9 are expressed in the human corpus luteum. MMP-2 and MMP-9 have previously been detected by zymography in homogenates of rat ovaries (Endo *et al.*, 1993a), bovine corpus luteum (Tsang *et al.*, 1995) and luteinised human granulosa-cells (Puistola *et al.*, 1995; Aston *et al.*, 1996a). MMP-1, MMP-2 and MMP-9 mRNAs have recently been described in the pseudo-pregnant rat ovary (Nothnick *et al.*, 1996). Collagen and other components of the ECM are an integral part of the structure of the corpus luteum (Luck and Zhao, 1995; Zhao and Luck, 1996). The human corpus luteum expresses enzymes with the capacity to proteolytically break-down these components of the ECM.

The expression and activity of MMPs in the corpus luteum changed during the luteal phase. MMP-2 expression and activity was maximal in the late-luteal corpus luteum. This is consistent with a role in tissue remodelling associated with luteolysis. In the rat, prolactin-induced structural luteolysis was associated with the activity of metalloproteinase enzymes, particularly MMP-2 (Endo *et al.*, 1993a). Interestingly, Aston *et al.* (1996a) have recently reported that MMP-2 activity increased with length of time of culture of luteinised granulosa cells. The

major MMP secreted from ovine luteal explants was MMP-2 (Russell *et al.*, 1995). Expression of MMP-2 in the corpus luteum may be associated with the tissue remodelling at the time of luteolysis.

In contrast, high levels of MMP-9 activity were also detected in the early-luteal phase. It is possible that MMP-9 is involved in the extensive tissue remodelling that occurs during the formation of the corpus luteum from the ruptured follicle (Luck and Zhao, 1995). A role of MMP-9 in the formation of the corpus luteum is supported by the finding that it is the primary metalloproteinase detected in follicle explants (Russell *et al.*, 1995). In addition, MMP-9 is the major MMP secreted into the culture medium of luteinised bovine (Zhao and Luck, 1996) and human granulosa cells (Puistola *et al.*, 1995; Aston *et al.*, 1996a). Dispersed luteal cells from four-day-old bovine corpora lutea had both MMP-2 and MMP-9 activity, but MMP-9 activity decreased with duration of culture (Tsang *et al.*, 1995) and MMP-9 was seen in the media of cultured human granulosa cells only during the first two days of culture (Puistola *et al.*, 1995). This provides preliminary evidence that MMP-9 may have a role in ovulation and the tissue remodelling associated with the formation of the corpus luteum.

When compared to the late-luteal phase, exposure of the corpus luteum to hCG during luteal 'rescue' was associated with reduced expression and activity of MMP-2. This is clearly different from the process of ovulation when LH/hCG stimulates an increase in MMP-1 and MMP-2 expression (Curry *et al.*, 1992; Tadakuma *et al.*, 1993; Hulboy *et al.*, 1997). Follicular levels of MMP-2 increase between the LH surge and ovulation (Russell *et al.*, 1995). In cultures of luteinised granulosa cells, hCG also was shown to reduce the expression of MMP-2 and MMP-9 (Aston *et al.*, 1996a; Stamouli *et al.*, 1996). Human granulosa cells cultured on a thin layer of ECM are lost from culture in the absence of gonadotrophin (Aston *et al.*, 1996a). These cells are released from culture, not as a result of cell death, but via an active process suppressed by hCG (Aston *et al.*, 1996b). One of the effects of hCG during maternal recognition of pregnancy appears to be the inhibition of metalloproteinase expression.

MMP-1 and MMP-2 had a similar cellular localisation in the human corpus luteum. They were expressed in the connective tissue stroma, the vascular pedicles and within the theca-lutein cell layer. Fibroblasts and endothelial cells are sources of MMPs (Woessner, 1991) and they are likely to express MMP-1 and MMP-2 in the corpus luteum. In the endometrium (Hampton and Salamonsen, 1994) and in

ovarian cancers (Naylor *et al.*, 1994) cells of the stroma also have been shown to express these enzymes. Although the expression of MMP-2 was maximal in the late-luteal phase, its localisation in the corpus luteum was not affected. This suggests the source for MMP during luteolysis is the periphery of the gland. In contrast, MMP-9 mRNA was localised to single cells in the steroidogenic and non-steroidogenic cell layers. The identity of these cells is uncertain but they are likely to be white blood cells. Polymorphonuclear leukocytes express MMP-9 (Murphy *et al.*, 1989) and we found that expression was often associated with blood vessels. Cells of the immune system, including macrophages, are found in the human corpus luteum (Brännström *et al.*, 1994a) and may be a source, or stimulator, of MMP expression.

It is unclear if MMPs are expressed by the granulosa-lutein cells of the corpus luteum. Few grains were localised to this cell layer and when present, they were in isolated individual cells. This finding is contrary to reports using cultures of luteinised granulosa cells (Puistola *et al.*, 1995; Aston *et al.*, 1996a). *In vitro* MMP-9 expression falls with continuing culture. This has led some authors to suggest that MMP-9 activity is related to leukocytes which accompany the granulosa cells in the first few days of culture (Puistola *et al.*, 1995). However, it is thought that bovine and human granulosa cells and bovine luteal cell dispersates in culture secrete MMP-2 (Tsang *et al.*, 1995; Puistola *et al.*, 1995; Zhao and Luck, 1996). Although it is possible that MMP-2 activity in these cultures results from white cell or thecal contamination, it is likely that granulosa-lutein cells have the potential to express MMPs and are induced to do so in culture. However, it is clear that the main site of MMP-2 expression in the corpus luteum is not the granulosa-lutein cells.

TIMP-1 and TIMP-2 have different cellular localisations in the corpus luteum. Comparison of serial sections immunostained for 17α-hydroxylase, to identify the luteal cells of thecal origin, showed that TIMP-2 was localised to the theca-lutein cells and the surrounding connective-tissue stroma. Smith *et al.* (1995a) found TIMP-2 in the theca of the ovine follicle. This is consistent with the primary localisation of TIMP-2 in the follicle being maintained in the mature corpus luteum. The localisation of TIMP-2 was similar to the localisation of MMP-1 and MMP-2. This suggests that TIMP-2 may have a role in the local regulation of these enzymes in the corpus luteum. Indeed, it has been suggested that TIMP-2 displays a preference for MMP-2 (Goldberg *et al.*, 1989). However, as we have

reported in **Chapter 10** (Duncan *et al.*, 1996c), the localisation of TIMP-1 is different. It is possible that TIMP-1 has other roles in addition to inhibition of metalloproteinases in the corpus luteum. It has been suggested that TIMP-1 has growth factor activity (Hayakawa *et al.*, 1992) and may function as a facilitator of steroidogenesis (Boujrad *et al.*, 1995; Duncan *et al.*, 1996b). However, the lack of significant ovarian disturbance in mice without a functional TIMP-1 gene (Nothnick *et al.*, 1997), means the role of high TIMP-1 expression in granulosa-lutein cells is not clear.

It was not clear how MMPs could function in the corpus luteum which expresses large amounts of the specific inhibitor, TIMP-1 (Juengel *et al.*, 1994; Duncan *et al.*, 1996c). We have shown that MMPs are expressed in different areas of the corpus luteum than TIMP-1. In addition, where MMPs were expressed in the granulosa-lutein cellular layer, the expression was localised to foci of individual cells. The localisation of MMPs seems to be a key factor in their activity in the corpus luteum.

The control of MMP expression in the corpus luteum is not clear. The detection and localisation of pro-MMP mRNA may not be related to the activity of MMPs. MMPs have to undergo enzymatic activation (Matrisian, 1990). The increased MMP-2 expression in the late-luteal phase parallels the increase in immune cells (Brännström *et al.*, 1994a). Immune cells may be the source of some MMPs, immune cell cytokines and chemoattractants may be involved in stimulation of MMP expression. MMP-9 is induced by IL-1 in the rat ovary (Hurwitz *et al.*, 1993), however IL-1 also stimulates TIMP expression (Nothnick and Curry, 1996). Prostaglandins, which are likely to be involved in the luteolytic process (Behrman *et al.*, 1993), may modulate MMP expression (Reich, 1991).

Steroid and trophic hormones may influence MMP expression (Hampton and Salamonsen, 1994; Salamonsen, 1996; Stamouli *et al.*, 1996; Hulboy *et al.*, 1997). Oestrogen induces a significant increase in MMP-2 activity *in vitro* (Puistola *et al.*, 1995). The primate corpus luteum does not express oestrogen receptors (Chandrasekher *et al.*, 1994) but does contain progesterone receptors (Chandrasekher *et al.*, 1994; Suzuki *et al.*, 1994). LH/hCG receptors are localised to different regions of the corpus luteum (Duncan *et al.*, 1996a) than MMPs. It is therefore likely that steroidogenic cell products inhibit the expression of MMPs in a paracrine fashion. The nature of these products is still unclear and more work is

required to dissect out the control of MMP expression and activity in the corpus luteum.

In conclusion, the expression of MMP-2 in the late-luteal phase may indicate a role for this enzyme in the tissue remodelling associated with luteolysis. One function of hCG during luteal 'rescue' is to prevent this increase in MMP expression. As TIMP-1 and TIMP-2 change little, it is likely that control of MMP activity in the corpus luteum involves changing MMP rather than TIMP expression. MMPs are localised in different areas than TIMP-1 and where they are expressed in the same area they are expressed in foci. This may explain how MMPs can function in the background of large amounts of TIMP-1.

Chapter 13

The human corpus luteum: reduction in macrophages during maternal recognition of pregnancy

13.1 Abstract

It has been shown that immune cells, particularly macrophages, accumulate in the corpus luteum during luteolysis. This study aimed to investigate the effect of maternal recognition of pregnancy on the localisation and numbers of macrophages in the human corpus luteum. Corpora lutea (n=12) were obtained from normally cycling women at the time of hysterectomy and were dated on the basis of serial urinary LH estimation. In addition, corpora lutea (n=4) were collected from women who had received daily doubling doses of hCG to mimic the hormonal changes of early pregnancy. Macrophages were localised by immunohistochemistry using an anti-CD68 antibody. Steroidogenic cells, steroidogenic cells of thecal origin and endothelial cells were identified on serial sections by immunohistochemistry for 3β-HSD, 17α-hydroxylase and von Willebrand factor respectively. The luteal cells capable of responding directly to hCG were identified by isotopic *in situ* hybridisation for mRNA encoding LH/hCG receptors. Macrophages were primarily localised to the vascular connective tissue and theca-lutein areas of the corpus luteum although some were found in the granulosa-lutein cell layer. Macrophage numbers increased throughout the luteal phase to a maximum in the late-luteal phase ($p<0.05$). Luteal 'rescue' with hCG was associated with a marked reduction in the numbers of tissue macrophages when compared to the late-luteal phase ($p<0.001$). One of the effects of hCG during maternal recognition of pregnancy is to prevent the normal influx of macrophages into the corpus luteum. As LH/hCG receptors localised to the steroidogenic cells, this implies a fundamental role for steroidogenic cell products in the control of macrophage influx into the human corpus luteum.

13.2 Introduction

In a non-conception cycle, the primate corpus luteum undergoes luteolysis with a loss of functional and structural integrity. The molecular events involved in luteolysis and how they are prevented by exposure to hCG during maternal recognition of pregnancy remain unclear (Behrman *et al.*, 1993). One feature of luteolysis, however, is the marked accumulation of immune cells in the corpus luteum. This increase in the number of immune cells has been reported in a variety of species, including rodents (Brännström *et al.*, 1994b), rabbits (Naftalin *et al.*, 1997), ruminants (Murdoch, 1987; Lei *et al.*, 1991), and women (Wang LJ *et al.*, 1992; Best *et al.*, 1996; Takaya *et al.*, 1997). As these species use disparate mechanisms to control their corpus luteum (Auletta and Flint, 1988), this common increase implies a fundamental role for immune cells, or their cytokine products (Brännström and Norman, 1993), in the luteolytic process.

The main immune cell to be localised in the human corpus luteum during luteolysis is the macrophage (Wang LJ *et al.*, 1992; Brännström *et al.*, 1994a; Best *et al.*, 1996). However, macrophage products have been shown to have both positive and negative effects on progesterone secretion. Macrophage products have been reported to have pro-steroidogenic effects in cell culture (Kirsch *et al.*, 1981; Kirsch *et al.*, 1983; Halme *et al.*, 1985). In addition, in the early stages of luteal function, macrophages are thought to have primarily luteotrophic effects (Brännström and Norman, 1993). Other macrophage products, however, such as TNFα, $PGF_{2\alpha}$, reactive oxygen species and NO, have been shown to have negative effects on steroidogenesis (Fairchild Benyo and Pate, 1992; Grusenmeyer and Pate, 1992; Vega *et al.*, 1995; Kato *et al.*, 1997; Van Voorhis *et al.*, 1994). Whereas macrophages are likely to be involved in the phagocytic clearance of cell debris (Paavola, 1979; Takaya *et al.*, 1997) after functional luteolysis, it is still not clear role tissue macrophages have in the functional corpus luteum.

We hypothesised that the primary role of macrophages in the human corpus luteum was luteolytic, rather than luteotrophic, and that macrophage numbers would not increase in the 'rescued' corpus luteum of early pregnancy. To test this hypothesis, we investigated the number of macrophages in the human corpus luteum throughout the functional luteal phase by immunohistochemistry. We compared the numbers of macrophages in corpora lutea in the late-luteal phase, where progesterone output is falling, with corpora lutea 'rescued' with exogenous

hCG, where progesterone output is increasing. We also hypothesised that any increase in macrophages within the corpus luteum was by *de novo* influx, rather than local changes in cellular localisation. To test this hypothesis, we identified the structural architecture of the corpus luteum by immunohistochemistry and compared the localisation of macrophages throughout the luteal phase and after luteal 'rescue' with exogenous hCG. In the final part of the study, we investigated the site of action of hCG on macrophages during luteal 'rescue' by localising LH/hCG receptors in the corpus luteum by isotopic *in situ* hybridisation.

13.3 Specific Materials and Methods

13.3.1 Tissues Studied

Corpora lutea were enucleated at the time of hysterectomy in 16 women undergoing surgery for benign conditions (**2.2**) and dated on the basis of serial urinary LH measurements (**2.2.4**). Four corpora lutea were classified as early-luteal, four as mid-luteal and four as late-luteal (**2.2.4**). Four 'rescued' corpora lutea were collected from women who had been treated with exogenous hCG to mimic the hormonal changes of early pregnancy (**2.2.2**). One piece of each corpus luteum was fixed in 4% paraformaldehyde (**2.2.3**) and embedded in paraffin wax for immunohistochemistry (**3.2**) and another piece was frozen in embedding medium to allow frozen sections to be prepared (**2.2.3**). In each case an endometrial biopsy was also fixed in paraformaldehyde and processed into paraffin wax for luteal phase dating by tissue morphometry (**3.2.8**).

13.3.2 Immunohistochemistry

Paraffin wax sections (5 μm) on poly-L-lysine-coated slides (**3.2.1**) were prepared (**3.2.2**). As preliminary experiments indicated that antigen retrieval using trypsinisation was necessary for the detection of the CD68 antigen and von Willebrand factor, this was then performed (**3.2.4**). Endogenous peroxidase activity was then blocked (**3.2.5**). Immunohistochemistry was performed as described (**3.2.6**) using NRS containing 4% (w/v) BSA to block non-specific binding. Sections were incubated with the primary antibody, monoclonal mouse anti-human macrophage CD68 antigen (**3.1.2**) diluted 1:50 in TBS, or the mouse anti-human von Willebrand factor (**3.1.2**) diluted 1:25, for one hour at room

temperature (Rodger *et al.*, 1997) (**3.2.6**). Mouse IgG at an equivalent antibody concentration was used as a negative control (**3.2.9**). Antibody binding was indicated by an AB-HRP reaction with a biotinylated rabbit anti-mouse secondary antibody at a dilution of 1:100 in TBS (**3.2.6**). The reaction was developed with DAB to give a stable brown end-product (**3.2.7**).

Steroidogenic cells were identified in serial sections by immunohistochemistry for 3β-HSD. Here, polyclonal rabbit anti-human 3β-HSD (**3.1.2**) was used in a dilution of 1:1000 (Riley *et al.*, 1992). Immunohistochemistry was performed as described above, without trypsinisation, using NGS to block non-specific binding (**3.2.6**) and biotinylated goat anti-rabbit immunoglobulins to detect specific binding (**3.2.6**). Steroidogenic cells of thecal origin were identified in serial sections by immunohistochemistry for 17α-hydroxylase as described previously (Rodger *et al.*, 1995) (**10.3.2**). Briefly, the polyclonal rabbit anti-human 17α-hydroxylase antibody (**3.1.2**) was used at a 1:750 dilution, NGS was used to block non-specific binding, and biotinylated goat anti-rabbit immunoglobulins were used as the secondary antibody. Rabbit serum with an equivalent immunoglobulin concentration was used as a negative control (**3.2.9**).

13.3.3 *In situ* Hybridisation

Isotopic *in situ* hybridisation for LH/hCG receptors was performed using antisense and sense ^{35}S-labelled riboprobes (**3.7**). The antisense probe, incorporating ^{35}S-labelled UTP was generated from the plasmid vector (**3.1.3**) linearised by HindIII (**3.5.3**) using T3 RNA polymerase (**3.7.2**). The ^{35}S-labelled sense probe was used as a negative control. This was generated from the plasmid vector (**3.7.1**) linearised by ECoR1 (**3.5.3**) using T7 RNA polymerase (**3.7.2**).

As preliminary experiments with fixed serial sections gave technically poor results, LH/hCG receptor mRNA was localised in frozen sections from the same corpora lutea. Frozen sections (**2.2.3**) were fixed, acetylated, dehydrated and dried under vacuum (**3.7.3**). One hundred microlitres of hybridisation buffer containing 1 x 10^6 c.p.m. radiolabelled riboprobe (**3.7.2**) was added to each section and the slides were incubated overnight at 55 °C in a moist chamber (**3.7.4**). The following day the slides were treated with RNAse A, and washed in increasingly stringent conditions (**3.7.5**). The sections were then dehydrated, allowed to dry and dipped in photographic emulsion (**3.7.6**). After incubation in the dark for 21 days, they were developed and fixed (**3.7.6**). The sections were then rinsed,

counter-stained with haematoxylin and mounted (**3.2.8**). They were viewed under dark-field illumination and the localisation of the silver grains was determined by reference to the image viewed under light-field illumination.

13.3.4 Analysis of Results

The number of macrophages was counted by an observer blinded to the tissue identity and repeated the following week to confirm the reproducibility of the results. Macrophage numbers in all sections during the repeat count were within 5% of the initial count. Macrophages were identified by intense brown staining on tissue sections. Only positive cells where the nuclei could be identified were counted in order to avoid counting tiny fragments of cells present in the tissue section. Sections were analysed using a stratified random sampling technique using a graticule lens. The stratified random sampling technique involved taking random fields from a grid of 24 fields arranged around a fixed, non-random, point (the centre of the section). At least five fields of each section were counted and the running mean was monitored to confirm adequate sampling. The granulosa-lutein cell layers, the theca-lutein cell layers and the surrounding stroma were identified by morphology and by comparison with serial sections immunostained for 3β-HSD and 17α-hydroxylase. In addition to the total number of macrophages, the number of macrophages in these layers was also recorded.

The number of macrophages at different stages of the luteal phase, and the number in the different cellular layers, were analysed by one way ANOVA with a 5% level of significance. Where significant differences were found to exist, pairwise comparisons using the Bonferroni/Dunn method were performed using commercial computer software (StatView 4.0).

13.4 Results

13.4.1 Plasma Progesterone Concentrations

The classification of the corpora lutea by serial urinary LH measurement agreed with the luteal-phase dating of endometrial biopsies using the method of Li *et al.* (1988). The plasma progesterone concentrations were 36.36 ±9.28 nmol/l in the early luteal samples, 40.35 ±9.88 nmol/l in the mid-luteal samples and 18.80

±12.81 nmol/l in the late luteal samples. After luteal 'rescue' by exogenous hCG the plasma progesterone concentrations had increased to 52.75 ±1.09 nmol/l.

13.4.2 Functional Anatomy of the Corpus Luteum

Steroidogenic cells were identified in human corpora lutea by the immunolocalisation of 3β-HSD (Fig. 13.1*a*). Luteal cells of both thecal and granulosal origin express this enzyme. The theca-lutein cells were specifically identified by immunolocalisation of 17α-hydroxylase (Fig. 13.1*b*). These cells formed clearly distinct populations, located around the peripheral margin of the granulosa-lutein cells. Vascular endothelial cells were localised by immunohistochemistry for von Willebrand factor (Fig. 13.1*c*). The theca-lutein cell layer had a rich blood supply. Endothelial cells were also scattered throughout the granulosa-lutein cell layers, particularly in the radial invaginations from the theca-lutein cell layer (Fig. 13.1*d*). LH receptors were localised by isotopic mRNA *in situ* hybridisation (Fig. 13.1*e*). As described previously, in **Chapter 5**, they were localised to the steroidogenic cell population (Duncan *et al.*, 1996a). When compared to immunostained sections, no hybridisation signal could be detected in endothelial cells, stromal cells or cells without the morphological characteristics of steroidogenic cells.

13.4.3 Localisation of Tissue Macrophages

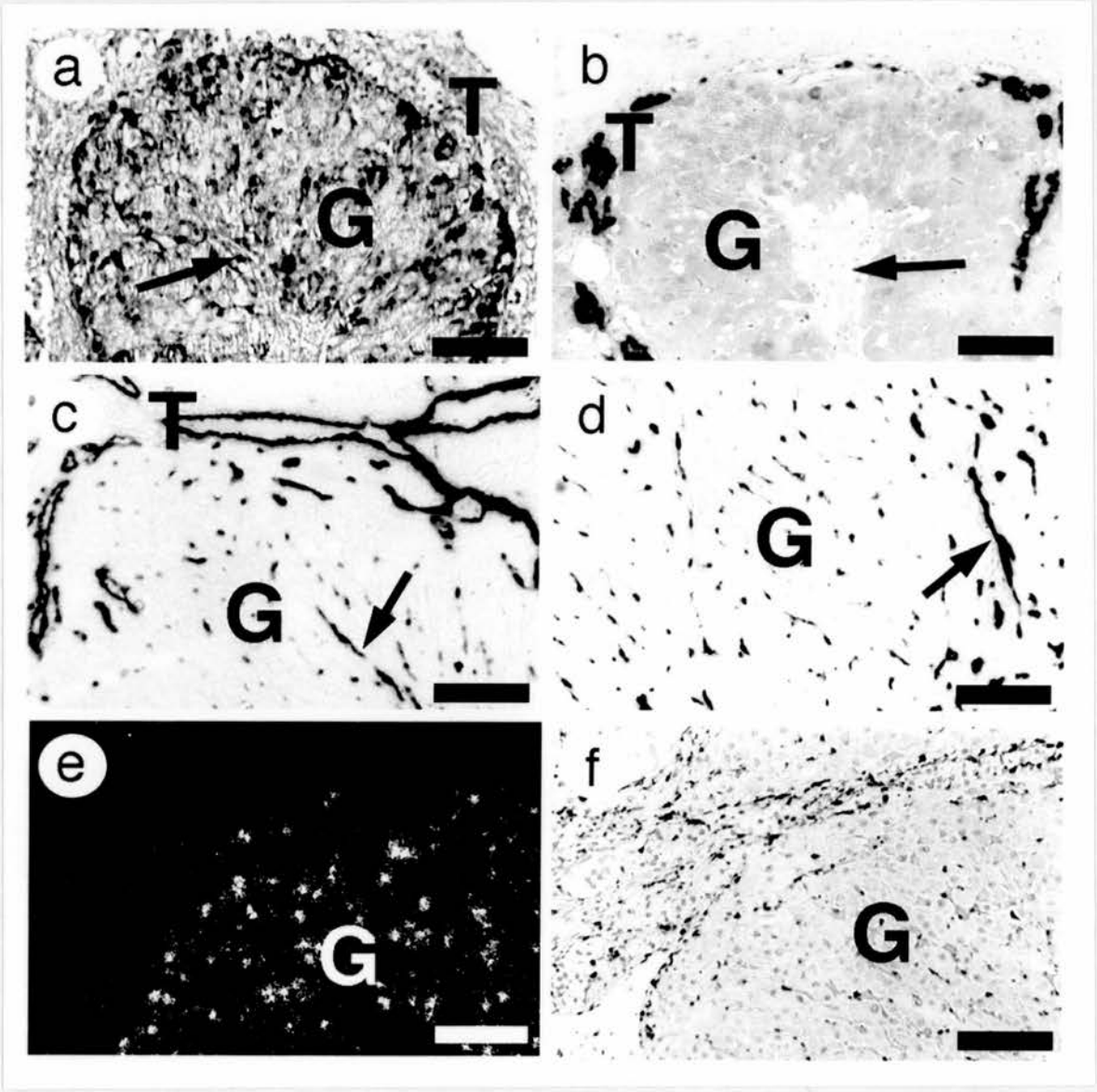
Macrophages, as described by immunohistochemical localisation of the CD68 antigen, could be localised in all corpora lutea studied (Fig. 13.2*a*). No staining was present in negative control sections where the primary antibody was replaced with an equivalent concentration of IgG (Fig. 13.2*b*). Many more macrophages could be detected in late-luteal corpora lutea (Fig. 13.2*c*) than after luteal 'rescue' with exogenous hCG (Fig. 13.2*d*). The numbers of macrophages in the corpora lutea at different stages of the luteal phase were counted. The macrophage content of the corpus luteum increased throughout the luteal phase reaching a maximum in the late-luteal phase ($p<0.05$) (Fig. 13.3). Luteal 'rescue' with hCG was associated with a reduction in the number of macrophages (Fig. 13.3), which were significantly lower that the late-luteal phase ($P<0.001$).

Macrophages were particularly prominent in the theca-lutein cell layer at all stages (Fig. 13.1*f*; Fig. 13.2*a*). In the granulosa-lutein cell layer, they were usually seen in association with the vascular in-foldings, particularly in the late-luteal

Figure 13.1

Functional anatomy of the human corpus luteum

Functional anatomy of the human corpus luteum: **a)** mid-luteal corpus luteum immunostained for 3β-HSD showing staining of the steroidogenic cells in the granulosa-lutein (G) and theca-lutein (T) cell layers and no staining of the connective tissue core (arrow); **b)** the same corpus luteum as (a) immunostained for 17α-hydroxylase showing staining in the theca-lutein (T) cells around the periphery of the granulosa-lutein cells (G), which, like the connective tissue core (arrow) show no immunoreactivity; **c)** the same corpus luteum as (a) immunostained for von Willebrand factor to demonstrate the endothelial cells which are abundant in the theca-lutein cell layer (T) and connective tissue invaginations (arrow) and also found in the granulosa-lutein cell layer (G); **d)** a closer view of the granulosa-lutein cell layer (G) of another mid-luteal corpus luteum immunostained for von Willebrand factor showing strong endothelial cell immunostaining in the vascular connective tissue invaginations (arrow); **e)** dark-field view of a mid-luteal corpus luteum after isotopic *in situ* hybridisation for LH receptor mRNA showing grains distributed over the granulosa-lutein cell layer (G); **f)** the same corpus luteum as (e) after immunohistochemistry for macrophages (CD68 +ve cells) showing the distribution of macrophages around the periphery of the granulosa-lutein cell layer (G), an entirely different localisation to LH receptor mRNA. Scale bar = 100 μm.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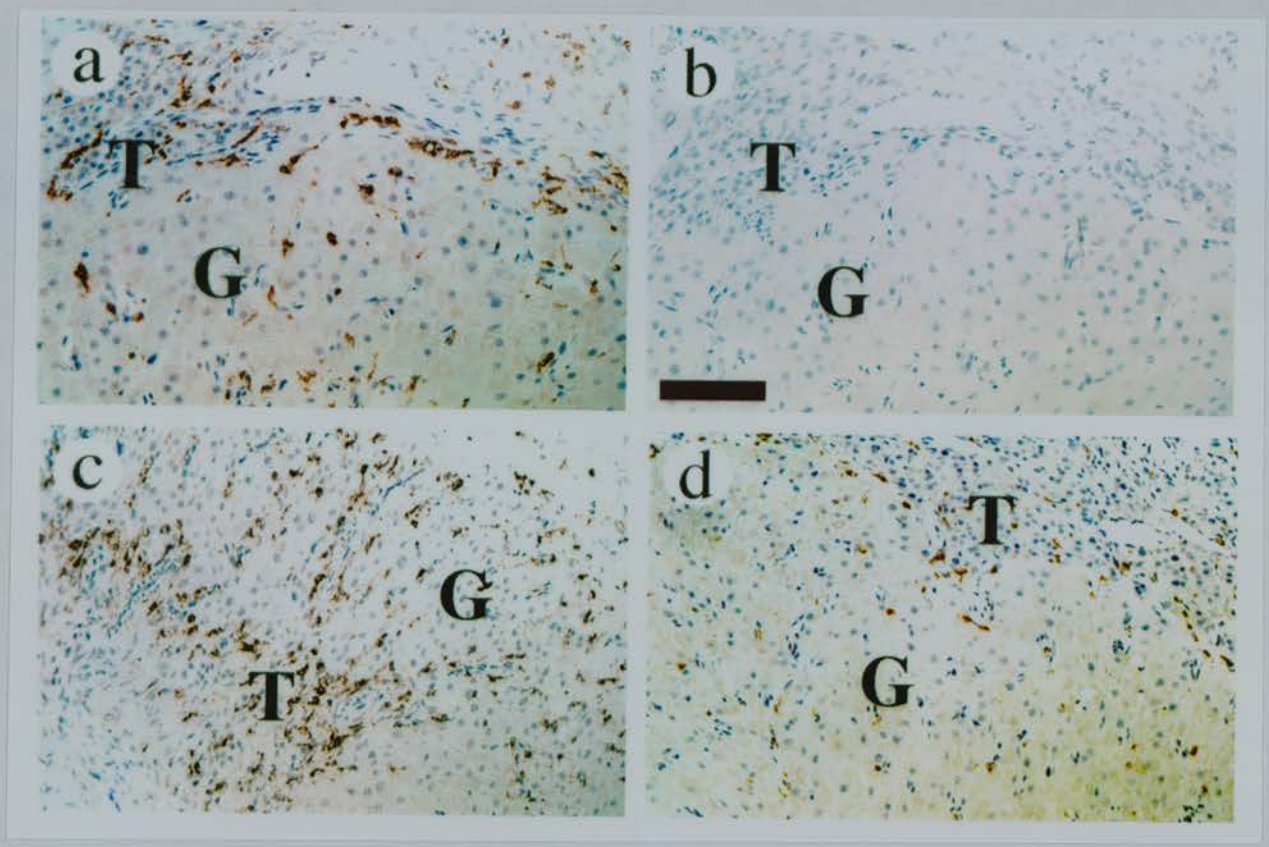

Figure 13.2

Localisation of macrophages in the human corpus luteum

Macrophage localisation in the human corpus luteum: **a)** mid-luteal corpus luteum with macrophages (CD68 +ve cells, brown) localised to the periphery of the steroidogenic cells in the theca-lutein layer (T) and also to the granulosa-lutein cell layer (G) and surrounding connective tissue stroma; **b)** negative control serial section of (a) showing the theca-lutein (T) and granulosa-lutein (G) cell layers with no specific staining visible; **c)** increased numbers of macrophages (CD68 +ve cells, brown) in the theca-lutein (T) and granulosa-lutein (G) cell layers in a late-luteal corpus luteum; **d)** corpus luteum after luteal 'rescue' with hCG showing fewer macrophages (CD68 +ve cells, brown) in the theca-lutein (T) and granulosa-lutein (G) cell layers. Scale Bar = 100 μm.

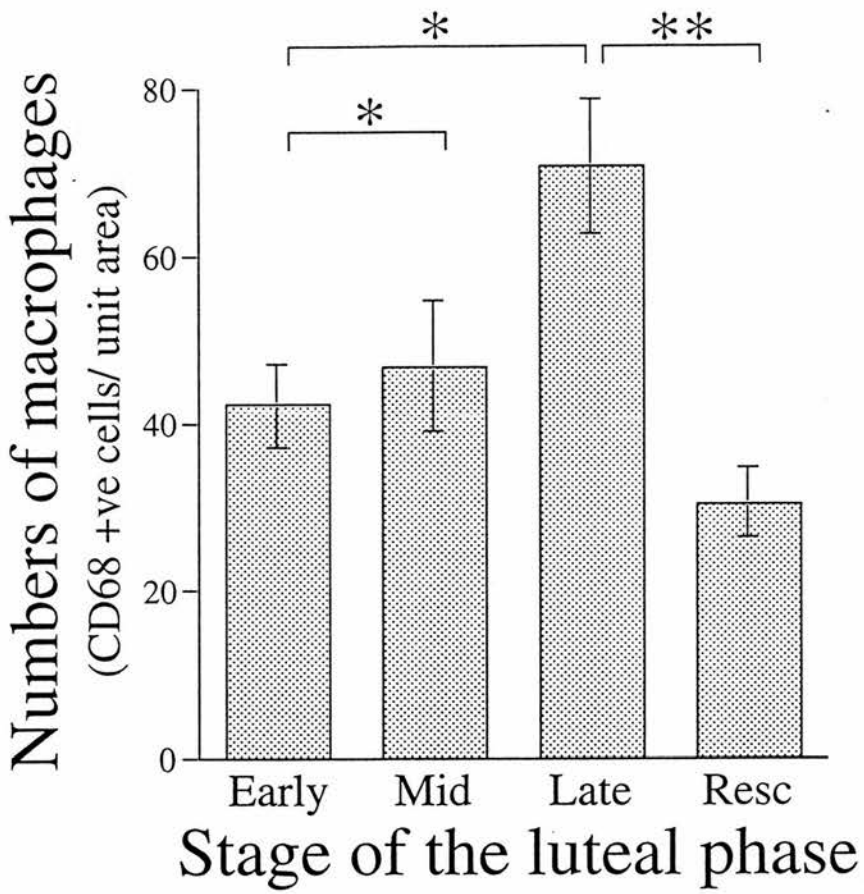

Figure 13.3

Numbers of macrophages in human corpora lutea

Macrophage numbers in corpora lutea: numbers of CD68 +ve cells in sections of human corpus luteum in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late- (LH+11 to LH+14) luteal phase and after luteal 'rescue' with hCG (hCGx5 to hCGx8). Values are mean ±S.E.M. (n=4 per group). Values were analysed by ANOVA and where significant differences at the 5% level were observed pairwise comparisons were conducted using the Bonferroni/Dunn method (*=$p<0.05$, **=$p<0.001$).

phase (Fig. 13.2*c*). The numbers of macrophages specifically located within the granulosa cell layer showed the same pattern as the overall macrophage content throughout the luteal phase (Fig. 13.4). There were no differences in the percentages of macrophages specifically located to the granulosa-lutein cell layer at any stage of the luteal phase. The localisation of the LH receptor (Fig. 13.1*e*) in the human corpus luteum was clearly different from the localisation of CD-68 positive tissue macrophages (Fig. 13.1*f*).

13.5 Discussion

We have compared the macrophage content in the corpus luteum of women who received exogenous hCG at concentrations equivalent to that of early pregnancy with corpora lutea obtained from untreated women at clearly defined stages of the luteal phase. We found that the macrophage content was significantly lower in the hCG-treated women than in the untreated women from the same stage of the luteal phase. This suggests that macrophage accumulation is associated with the loss of luteal integrity during luteolysis and that one of the effects of hCG during luteal 'rescue' is to prevent the accumulation of macrophages in the corpus luteum.

In this study we have concentrated on the numbers and localisation of macrophages in the corpus luteum. Various other immune cells have also been identified in the corpus luteum, including polymorphonuclear leukocytes (Brännström *et al.*, 1994a) and T-cells (Best *et al.*, 1996). However, it is clear that macrophages are the predominant immune cell subpopulation in the human and rabbit corpus luteum (Wang LJ *et al.*, 1992; Best *et al.*, 1996; Bagavandoss *et al.*, 1990). In addition, unlike other immune cells, macrophages have clearly been shown to vary in number during the lifespan of the corpus luteum, being particularly abundant in the regressing corpus luteum (Best *et al.*, 1996; Brännström *et al.*, 1994a). Although other immune cells may be affected during luteal 'rescue', it is likely that the clearest and most predominant effect is that on tissue macrophage content.

Our study confirms that macrophage influx increases during the functional lifespan of the corpus luteum. Macrophage accumulation in the corpus luteum is a feature of luteolysis in a variety of animal species, including rats (Brännström *et al.*, 1994b), rabbits (Bagavandoss *et al.*, 1990; Naftalin *et al.*, 1997), pigs (Hehnke

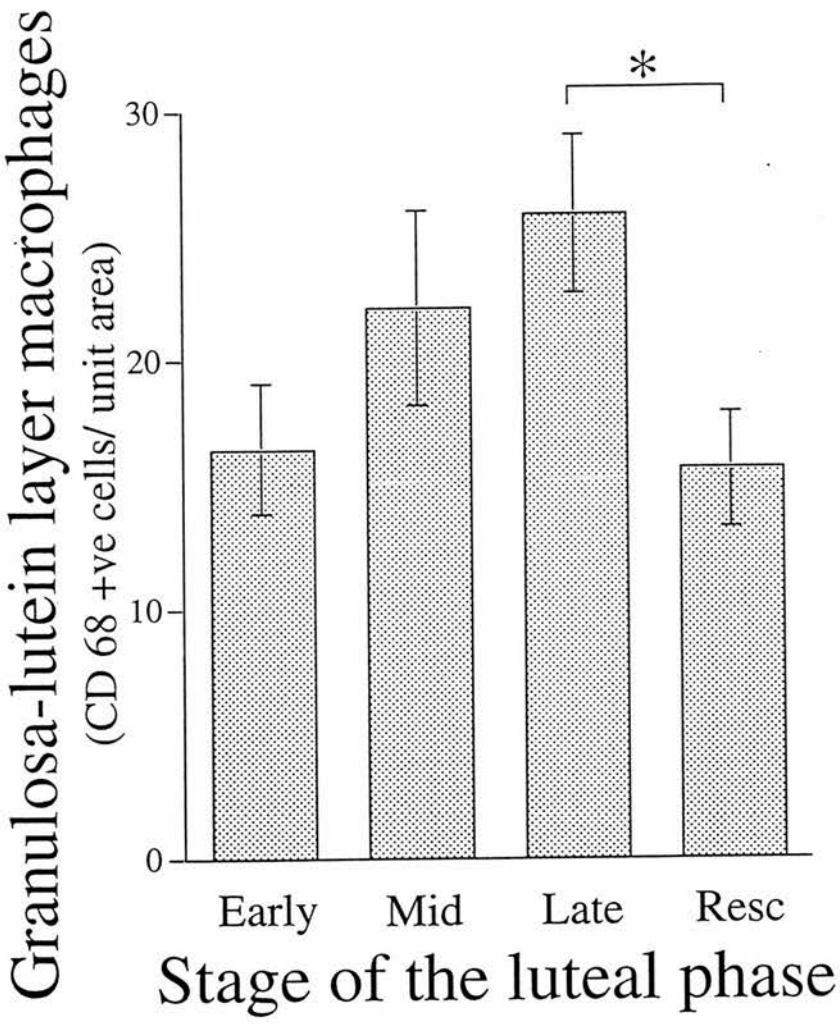

Figure 13.4

Numbers of macrophages in the granulosa-cell layer of human corpora lutea

Macrophage numbers within the granulosa-lutein cell layer: numbers of CD 68 +ve cells in sections of human corpus luteum in the early- (LH+1 to LH+5), mid- (LH+6 to LH+10) and late- (LH+11 to LH+14) luteal phase and after luteal 'rescue' with hCG (hCGx5 to hCGx8). Values are mean ±S.E.M. (n=4 per group). Values were analysed by ANOVA and where significant differences at the 5% level were observed pairwise comparisons were conducted using the Bonferroni/Dunn method (*=$p<0.05$).

et al., 1994), sheep (Murdoch, 1987) and women (Brännström *et al.*, 1994b; Best *et al.*, 1996; Takaya *et al.*, 1997). Macrophages are clearly present in large numbers in the regressing corpus luteum after menstruation in women (Wang LJ *et al.*, 1992; Brännström *et al.*, 1994a; Takaya *et al.*, 1997). However, there has been some debate about the accumulation of macrophages in the late-luteal phase when the corpus luteum is still producing progesterone. Brännström *et al.* (1994a) failed to find an increase in luteal macrophages in the late-luteal phase. In contrast, other studies (Lei *et al.*, 1991; Best *et al.*, 1996) reported increased macrophages in the functional corpora lutea in the late-luteal phase. It is likely that the discrepant findings of Brännström *et al.* (1994a) are explained by their more extended definition of the late-luteal phase (from LH+8) and that macrophage influx is a feature of luteal ageing.

The role of macrophage accumulation in the late-luteal phase is not fully established. It is not clear whether it is a cause or consequence of falling progesterone synthesis. Clearly macrophage products can inhibit steroidogenesis *in vitro*. NO (Van Voorhis *et al.*, 1994), TNFα (Fairchild Benyo and Pate, 1992; Wang HZ *et al.*, 1992), $PGF_{2\alpha}$ (Grusenmeyer and Pate, 1992), reactive oxygen species (Vega *et al.*, 1995; Kato *et al.*, 1997) and interleukins (Sjögren *et al.*, 1991) have all been shown to inhibit the steroidogenic pathway. In addition, cell death by apoptosis is a feature of luteolysis in many species (Dharmarajan *et al.*, 1994; Zheng *et al.*, 1994; Shikone *et al.*, 1996) and apoptosis can be promoted by macrophage products, such as free oxygen radicals, TNFα and some interleukins (Hale *et al.*, 1996; Jacobson, 1996; Spencer *et al.*, 1996). Macrophages may have a role in structural luteolysis, they can clear cellular debris by phagocytosis (Paavola, 1979) and activate the MMP enzymes (Hurwitz *et al.*, 1993; Hulboy *et al.*, 1997) which have been implicated in the remodelling associated with luteolysis (Endo *et al.*, 1993a; Luck and Zhao, 1995). Whether it is cause or consequence of falling progesterone synthesis, it is likely that the accumulation of macrophages in the late-luteal phase has a negative effect on the structure and function of the corpus luteum.

Macrophages however have been shown to have both pro-steroidogenic and luteotrophic properties under some conditions. It remains possible that the macrophage accumulation in the functional luteal phase is a luteotrophic response to failing progesterone synthesis. Macrophage-derived products have been shown to enhance progesterone output from luteal cells in culture (Kirsch *et al.*, 1983;

Halme *et al.*, 1985), and macrophages may secrete factors important for angiogenesis and tissue reorganisation. Brännström and Norman (1993) postulated a luteotrophic effect of macrophages in the early luteal phase. In addition, as macrophages could enhance progesterone synthesis (Kirsch *et al.*, 1981; Kirsch *et al.*, 1983) and promote proliferation of granulosa cells (Fukumatsu *et al.*, 1992), Bukovský *et al.* (1995) also proposed a luteotrophic role of macrophages. In the rat corpus luteum, non-steroidogenic cells, probably white blood cells, have also been shown to have potent stimulatory effects on luteal cell steroidogenesis (Nelson *et al.*, 1992). It is therefore not clear whether the accumulation of macrophages in the corpus luteum would always be associated with a fall in progesterone synthesis.

We have demonstrated that luteal 'rescue' with exogenous hCG to mimic the early stages of pregnancy is associated with a reduction in the numbers of macrophages in the late-luteal corpus luteum. This supports the hypothesis that macrophages have a primarily luteolytic, rather than luteotrophic role, in the human corpus luteum. Our findings are different to those of some sub-primate species. Brännström *et al.* (1994b) found particularly high concentrations of macrophages in the rat corpus luteum during the early stages of pregnancy and pseudopregnancy. They found six-fold more macrophages in the corpus luteum of early pregnancy than during luteolysis (Brännström *et al.*, 1994b). This accumulation of macrophages is also seen in the corpus luteum of pregnant rabbits (Bagavandoss *et al.*, 1990). In the rabbit corpus luteum, oestrogen withdrawal induces macrophage invasion, but subsequent oestrogen replacement maintained progesterone production and did not necessarily reduce macrophage numbers (Naftalin *et al.*, 1997). Indeed, in that model system, the relative numbers of macrophages had no apparent relationship to progesterone synthesis. They concluded that the presence of macrophages did not preclude the continuation of progesterone production (Naftalin *et al.*, 1997). It is not known whether the human corpus luteum can continue to function in the presence of increasing numbers of macrophages, but clearly, macrophage influx is not a feature of luteal 'rescue' with hCG.

What promotes the influx of macrophages into the failing corpus luteum? Several chemoattractant and macrophage stimulatory molecules have now been identified, including cytokines such as IL-8 (Norman and Brännström, 1994), granulocyte-macrophage colony stimulating factor (GM-CSF) (Nicola, 1989) and monocyte

chemoattractant protein 1 (MCP-1) (Leonard and Yoshimura, 1990). These molecules can be detected in the ovary (Robertson and Seamark, 1990; Zhao *et al.*, 1995; Arici *et al.*, 1997). Recently, Townson *et al.* (1996) reported increased expression of MCP-1 in the corpus luteum during luteal regression in rats. This increase preceded the appearance of macrophages in the corpus luteum and they concluded that MCP-1 may have a prominent role in the immunological process of luteal regression. Induction of structural luteolysis by prolactin in rat corpus luteum (Bowen *et al.*, 1996), and by oestrogen withdrawal in the rabbit corpus luteum (Naftalin *et al.*, 1997), is associated with macrophage accumulation and expression of MCP-1. MCP-1 can be stimulated by cytokines (Oppenheim *et al.*, 1991; Arici *et al.*, 1997) which can be found in the corpus luteum during luteolysis (Brännström and Norman, 1993). However, MCP-1 expression can also be stimulated by hCG in granulosa-lutein cell culture (Arici *et al.*, 1977). Data on the expression of these chemoattractant molecules in the human corpus luteum throughout the luteal phase is not yet available, but would clearly be of great interest.

How does exposure to hCG during luteal 'rescue' prevent the influx of macrophages into the corpus luteum? HCG exerts its biological actions by binding to, and activating, LH receptors (Cole *et al.*, 1973). LH receptors are localised to the steroidogenic cells of the corpus luteum (Nishimori *et al.*, 1995; Duncan *et al.*, 1996a) and we have shown the localisation of these receptors is different to the localisation of macrophages. Although we were not able to co-localise macrophages and LH receptors on the same tissue section, it is unlikely that macrophages themselves express the LH receptor. The effect of hCG on macrophage accumulation therefore seems to be mediated through the steroidogenic cells. This effect is likely to be associated with the production or withdrawal of steroid or non-steroid molecules from the cells expressing LH/hCG receptors. Progesterone itself may function as a signal molecule as progesterone receptors can be localised to the human corpus luteum (Suzuki *et al.*, 1994). While these receptors appear to be present on steroidogenic cells (Suzuki *et al.*, 1994; Hild-Petito and Fazleabas, 1997), they can also be found on other cells within the connective tissue stroma (Suzuki *et al.*, 1994). It is not known whether luteal macrophages express progesterone receptors or whether luteal sex steroids can directly affect migration.

In our study, macrophages were found associated with the theca-lutein cell layer and the vasculature of the corpus luteum. An early study reported that macrophages were predominant in the granulosa-lutein cell layer of the human corpus luteum (Gillim *et al.*, 1969). However, later studies found a predominance in the theca-lutein layer (Wang LJ *et al.*, 1992; Brännström *et al.*, 1994a). We have used steroidogenic markers to confirm this observation. Brännström *et al.* (1994a) reported that macrophages were more abundant in the theca-lutein layer and were particularly associated with blood vessels. The relationship with blood vessels may suggest a recruitment of monocyte/macrophages from the circulation. In our study macrophage numbers increased during the late-luteal phase, in all cellular compartments. This is consistent with recruitment from the circulation rather than local migration. Interestingly, MCP-1 is secreted by several cell types including endothelial cells and fibroblasts (Leonard and Yoshimura, 1990). In the rat corpus luteum, luteal vascular cells appear to be a source of MCP-1 (Townson *et al.*, 1996). The endothelial cells and their communication with the steroidogenic cells of the corpus luteum may play a major role in the control of macrophage recruitment.

In conclusion, this study has shown that macrophages accumulate in the human corpus luteum during the luteal phase and are maximal in the late-luteal phase. One of the effects of hCG during luteal 'rescue' is to prevent this influx of macrophages into the corpus luteum. As macrophages do not express LH/hCG receptors this effect is mediated indirectly through factors produced by the steroidogenic cells. Further work is needed to study the expression of chemoattractant molecules in the human corpus luteum throughout the luteal phase and after luteal 'rescue' with hCG.

Chapter 14

General Discussion

This thesis has presented the results of a series of investigations into the properties of the primate corpus luteum at different stages of its functional lifespan. These results are contained in the eight experimental chapters. Each chapter has been deliberately written in the form of a complete scientific paper that can be read in isolation. Each chapter, therefore, ends with a specific discussion and a set of conclusions. The role of this chapter is not to rehash these specific discussions and conclusions but to examine the results, in the context of the whole thesis, in order to attempt to synthesise a speculative paradigm for the molecular mechanisms involved in the control of the human corpus luteum.

This chapter begins with a careful examination of the experimental models utilised. Clearly, an extrapolation of the results to normal primate luteal physiology depends of the validity of the models used. The next part of the discussion is concerned with potential improvements in experimental design that would allow additional information to be collected. In the next section, the conclusions of the experimental work in this thesis are revisited and an attempt made to unify them in an extended paradigm for primate luteal function. This raises further questions and areas of study which are then addressed and a strategy for the further investigation of the corpus luteum is developed. This chapter will hopefully form the introduction to a continuing programme of research in the human corpus luteum.

14.1 Are the Models Valid?

14.1.1 General Validity

What is the rationale for studying the corpus luteum? There are several answers to this question, as the corpus luteum has properties of general interest to cell biologists and specific interest to reproductive biologists. The human corpus luteum is a very dynamic gland, with high levels of natural cellular growth, death, angiogenesis and tissue remodelling (Behrman *et al.*, 1993). Understanding how

these processes occur, and are controlled, is likely to have direct relevance to other dynamic tissue processes including neoplasia, wound healing and menstruation. In addition, the corpus luteum is a highly active steroidogenic gland (Zeleznik and Fairchild Benyo, 1994). Understanding how luteal cells achieve such high levels of steroidogenesis, at a molecular level, may give us insights about general cellular metabolism, relevant to other endocrine and non-endocrine cell types. The corpus luteum is a valuable resource that is likely to have validity in a number of fields outwith general reproductive biology.

14.1.2 Specific Validity

General validity aside, the main rationale for these studies was the investigation of the molecular mechanisms important during the lifespan of the corpus luteum itself. Clearly, these studies aimed to broaden and expand our knowledge of the physiology of the corpus luteum. The reason that we want to specifically understand how the corpus luteum works, is that an increased understanding of how it functions may facilitate the development of novel approaches to the prevention or promotion of human fertility. The validity of all the conclusions reached in this thesis depends on how representative the models used are of normal luteal physiology. One of the most important aspects of luteal models, therefore, relates to their direct relevance to the human.

Most reported studies on the corpus luteum use rodent, rabbit or ruminant models (Niswender *et al.,* 1985; Auletta and Flint, 1988; Behrman *et al.,* 1993; Niswender and Nett, 1994). These models are convenient, accessible, easily manipulated, and in some cases of direct agricultural relevance (Niswender *et al.,* 1985). While it is likely that the molecular characteristics of different mammalian corpora lutea have many common elements, it is clear that there are many fundamental differences between infraprimate and primate corpora lutea (Auletta and Flint, 1988; Niswender and Nett, 1994; Zeleznik and Fairchild Benyo, 1994). Several of these major structural, regulatory and functional differences have already been discussed in this thesis. Whereas important insights into the molecular mechanisms involved in the control of primate luteal function can be gained by studying infraprimate species, the nature of the primate corpus luteum can only be elucidated by studying primate tissue. The major relevance of these studies, when trying to understand the human corpus luteum, is that human and non-human primate models were utilised.

The use of human models to study the primate corpus luteum is not unique by any means. Many observational studies on human luteal tissue have been reported (Bramley *et al.*, 1987; Bukovský *et al.*, 1995; Nishimori *et al.*, 1995; Shikone *et al.*, 1996; Best *et al.*, 1996), although when compared with the number of studies on infraprimate and non-human primate species, human tissue is studied uncommonly. Indeed, because of the difficulty in obtaining human corpora lutea, small numbers are often studied, and most of these studies report the use of archival corpora lutea, or corpora lutea collected on an ad-hoc basis. One of the problems with previous human studies is the lack of facilities for the systematic collection of corpora lutea.

14.1.3 Accurate Dating of Corpora Lutea

One of the strengths of the studies reported in this thesis (Koh *et al.*, 1995; Duncan *et al.*, 1996a; Duncan *et al.*, 1996b; Duncan *et al.*, 1996c; Duncan *et al.*, 1998a; Duncan *et al.*, 1998b; Duncan *et al.*, 1998c) is that all the corpora lutea investigated had been carefully dated. For the first time, the stage of the luteal phase has been accurately defined, on the basis of the urinary LH peak. In addition, only corpora lutea in which the urinary LH peak agreed with the LMP, and the morphological luteal phase dating (Li *et al.*, 1988) were used. This has ensured a robust and valid classification of functional human corpora lutea. As no other human studies have been as rigorous in luteal phase dating and classification, it is likely that the studies reported here are at least as valid as previous observational human studies. Indeed, it is possible that less robust dating measures could skew observational results obtained throughout the menstrual cycle. This suggests that the human model system reported in this thesis is as valid as possible in the classification of luteal phase.

14.1.4 Are these Corpora Lutea Representative?

It is reasonable to conclude that the results in this thesis are reliable, as far as the validity of the model system and the robust nature of the dating classification are concerned. However, their relevance to normal luteal physiology depends on how representative of normal, the human corpora lutea were. Clearly, the corpora lutea were obtained from women with gynaecological problems, and this is a potential source of bias. The selection criteria tried to minimise this bias by excluding those with systemic disease, those exposed to recent hormonal preparations and those

with a history of infertility. In addition, all women studied had regular menstrual cycles, which implies normal folliculogenesis and ovulation (Cowan, 1997). However, these women did require hysterectomy for benign conditions. It is possible that the group of patients undergoing surgery were in some way different from the normal fertile population, or that the pathological process, that necessitated surgery, affected the corpus luteum. It is also possible that the stress involved in the run up to surgery, augmented the release of factors which directly or indirectly altered the corpus luteum.

There are other potential sources of bias. The women in these studies tended to be older, and towards the end of their reproductive life, as well as having benign gynaecological disease. There is some evidence for subtle and overt ovarian dysfunction increasing with age, and being present in women with benign gynaccological conditions (Stouffer, 1990; Olive, 1991; Dawood, 1994; Bukovský *et al.*, 1996). It remains possible that the results were biased by these factors. However, the women were all ovulatory with normal menstrual cycle length. Although the clinical relevance of luteal dysfunction has been widely debated (Balasch and Vanrell, 1987; Olive, 1991; Jones, 1991; Dawood, 1994; Hinney *et al.*, 1996), there is no convincing evidence for luteal dysfunction, in the presence of normal folliculogenesis, in normally cycling fertile women. Indeed, these women had all been pregnant, implying normal luteal function in the past. It is likely that the tissues studied were as representative as possible of normal human corpora lutea.

14.1.5 Other Human Models

Since the advent of assisted conception, luteinised granulosa cells have been increasingly used to study luteal function. As these cells produce progesterone in culture, and exhibit a dose-response stimulation with hCG, they have been advocated as a model system to study the corpus luteum (Soto *et al.*, 1984; Wang *et al.*, 1991; Aston *et al.*, 1996a; Stamouli *et al.*, 1996). While these models have proven useful, and increased our understanding of the molecular mechanisms of luteal function (Abayasekara *et al.*, 1993; Endo *et al.*, 1993b), their use is limited. This is because, cellular connections, transient cells, supporting matrix, fibroblasts and theca-lutein cells, in correct anatomical relationships, are missing. All these elements are thought to contribute to normal luteal function (Behrman *et al.*, 1993; Zeleznik and Fairchild Benyo, 1994). The same problems are also

present when cultures of luteal cells (Stouffer *et al.*, 1977; Brannian *et al.*, 1992; Devoto *et al.*, 1995) are studied. Another approach to the *in vitro* analysis of the cellular function of luteal cells has been to use luteal slices (Girsh *et al.*, 1996; Hagstrom *et al.*, 1996; Fairchild Benyo and Zeleznik, 1997). This has the advantage of maintaining tissue architecture and cellular communications. However, the extent to which the tissue response is modified by mechanical sectioning is unknown. It is likely that luteinised granulosa cell, luteal cell and luteal slice cultures are valuable tools, but it has to be accepted that data interpretation is difficult, and the results have to be validated in whole corpora lutea.

14.1.6 Non-Human Primate Models

Most of our understanding of the primate corpus luteum has come from observational, functional and interventional studies involving new world monkeys (Stouffer, 1988; Behrman *et al.,* 1993). There is little evidence that these species differ significantly from the human in terms of their ovarian cycle. Monkey models allow careful dating and collection of tissue. However, the numbers used tend to be low, for humanitarian and expense reasons. In addition, all observations require to be tested on the human to ensure their complete validity. Primate models have proved very useful (Hutchison and Zeleznik, 1984; Hutchison *et al.*, 1986; Stouffer, 1988; Duffy *et al.*, 1994; Auletta and Kelm, 1994), particularly where interventions are required. However for observational studies, it is likely that human tissue represents the most appropriate investigative modality.

14.1.7 Maternal Recognition of Pregnancy

As enucleation of corpora lutea in the early stages of pregnancy causes miscarriage (Csapo *et al.*, 1973), it is unethical to collect corpora lutea from early human pregnancy. In the past, human luteal tissue from ectopic pregnancies has been studied (Rao *et al.*, 1977b; Bramley *et al.*, 1987; Dawood and Khan-Dawood, 1994; Nishimori *et al.*, 1995). However, this tissue is unlikely to be representative of normal early pregnancy, as the diagnosis is made at a gestation when placental progesterone is becoming increasingly important (Hearn, 1986; Zeleznik and Fairchild Benyo, 1994), and hCG production, and luteal function, is failing (Barnea *et al.*, 1986; Ledger *et al.*, 1994; Duncan *et al.*, 1995). This problem has been tackled in the past using primate models. Corpora lutea can be

collected from monkeys in the early stages of pregnancy and studied (Webley *et al.*, 1990; Hild-Petito and Fazleabas, 1997). In addition, exogenous hCG can be administered to 'rescue' the corpus luteum (Ottobre and Stouffer, 1986; Stouffer *et al.*, 1987; Christenson and Stouffer, 1996a). Such studies have given valuable insights but their use has been limited to the non-human primate (Stouffer, 1988).

A model has been developed, and validated, to 'rescue' the corpus luteum and mimic the hormonal changes of early pregnancy in women (Illingworth *et al.*, 1990). This model has been used for the first time in the studies reported in this thesis, to study the molecular effects of maternal recognition of pregnancy in the human corpus luteum. Its novelty clearly makes this tool a unique and valuable addition to the investigation of the human corpus luteum. Its validity is assured, as far as the accurate simulation of the hormonal changes of early pregnancy is concerned (Illingworth *et al.*, 1990; Illingworth *et al.*, 1996). However, its specific validity depends on whether it is only hCG that is involved in luteal 'rescue' and pregnancy recognition. Some authors have postulated that other molecules from the uterus or embryo are involved (Johnson *et al.*, 1993; Lower *et al.*, 1993). However, the available evidence is limited, and most researchers accept, as far as current data are concerned, that hCG appears to be the sole factor involved in maternal recognition of pregnancy in higher primates (Behrman *et al.*, 1993; Zeleznik and Fairchild Benyo, 1994).

14.1.8 Induced Luteolysis in the Marmoset

How representative is the marmoset model of induced luteolysis? There are several areas which should be discussed in relation to this. The first concerns how representative the marmoset is of normal primate physiology. The marmoset is a small primate with an ovarian cycle similar to higher primates. There are some differences however. The length of the marmoset luteal phase tends to be longer than the human (Lunn, 1998). In addition, the marmoset monkey often ovulates two follicles (Hearn *et al.*, 1978). The proportion of luteal tissue in the ovary during the luteal phase is greater than that seen in women (Webley *et al.*, 1990). Indeed, there are some differences in the microanatomy of the corpus luteum. Specific theca-lutein and granulosa-lutein cells are difficult to identify (Webley *et al.*, 1990), and marmoset ovaries contain accessory areas of luteinised tissue which are steroidogenically active (Fraser *et al.*, 1995a; Torii *et al.*, 1996), and may behave differently to corpora lutea. It is not clear if structural luteolysis is as

intimately linked to functional luteolysis in the marmoset (Young *et al.*, 1997), as it is in the human (Corner, 1956).

These concerns mean that it is not clear how representative the marmoset is of normal human ovarian physiology. However, the marmoset is a primate, and its corpus luteum appears to be controlled in the same way during the luteal phase (Lunn, 1998), during maternal recognition of pregnancy (Hearn and Webley, 1987) and, as far as we can tell, during functional luteolysis, as the human. Although, the model has some deficiencies, it is a more valid model than infraprimate species. Where the marmoset has its benefit, is in its size, ease of husbandry, time to ovarian maturity (Hearn *et al.*, 1978; Lunn, 1998), and ease of luteolytic manipulation (Webley *et al.*, 1991; Fraser *et al.*, 1995b). Luteolysis can easily be induced in the marmoset (Webley *et al.*, 1991), and this gives us the potential of studying closely co-ordinated changes. In addition, the fact that disparate mechanisms can be used to induce luteolysis (Webley *et al.*, 1991), ensures that the effects are not specific to one treatment modality.

How representative of natural luteolysis is induced luteolysis? Things are clearly different. In infraprimate species, induced luteolysis in frequently used in the study of the corpus luteum (Ji *et al.*, 1991; Endo *et al.*, 1993a; Hehnke *et al.*, 1994; Smith *et al.*, 1996b). However, due to species difference in the control of luteolysis (Auletta and Flint, 1988), in these species, induced luteolysis mirrors more closely what happens naturally. Induced luteolysis induces cell death and morphological changes (Fraser *et al.*, 1995b; Fraser *et al.*, 1995c; Young *et al.*, 1997) in the marmoset. These are very dramatic, but recent studies have shown that early follicular corpora lutea, in advanced stages of luteolysis, show similar levels of cell death and morphological changes, albeit less marked (Young *et al.*, 1997). Although differences clearly exist, there appear to be parallels of induced luteolysis with natural luteolysis (Auletta *et al.*, 1995). The model can be criticised, but there are advantages in studying co-ordinated luteolysis, and as far as induced luteolysis in the primate is concerned, the marmoset is the most studied primate model. No alternative, or more valid models, are regularly used or are currently available.

14.1.9 Conclusions

In conclusion, the models can be criticised. However, as far as human tissue is concerned, the model used is at least as valid as other human studies and offers

the advantage of accurate dating. In addition, the use of luteal 'rescue' with exogenous hCG gives a novel and exciting mechanism to systematically study the corpus luteum of early pregnancy in women for the first time. The bank of human corpora lutea is unparalleled elsewhere as a model system. There are more deficiencies with the marmoset model. However, the model of induced luteolysis in the marmoset is well defined (Webley *et al.*, 1991; Fraser *et al.*, 1995b) and, currently, is the most used model for primate luteolysis available. The models studied are at least as valid as the models used in the past, from which our current understanding of primate luteal physiology are derived.

14.2 Improvements in the Collection of Tissue

While the research reported in this thesis was underway, it became clear that improvements were possible in the collection of tissue, and in the types of tissue collected. It is likely that such improvements would increase the information obtained from the molecular investigations. However, in order to ensure a comparable set of tissues, collected with the same protocols, these improvements were not incorporated into the protocols. However, the following changes should be considered for future, or continuing studies.

14.2.1 Collection of Menstrual Corpora Lutea

One of the deficiencies in these studies is the lack of menstrual corpora lutea. Clearly, luteolysis is a dynamic process, and although functional luteolysis is complete at the end of the luteal phase, it is likely that remodelling continues through the early follicular phase. Previous studies have looked at menstrual corpora lutea on an ad-hoc basis (Ravindranath *et al.*, 1992a; Brännström *et al.*, 1994a; Nishimori *et al.*, 1995; Best *et al.*, 1996). It has been shown that regressive changes can be seen in early follicular corpora lutea (Young *et al.*, 1997). Although, the data collected on functional corpora lutea are clearly of primary importance, studies on menstrual corpora lutea have the potential to add to the picture, particularly when studying regression. In the future, it is suggested that early follicular corpora lutea should also be collected.

The collection of menstrual, or early follicular, corpora lutea, however, may be more difficult than it sounds. During the late-luteal phase, there is marked

remodelling of the corpus luteum (Corner, 1956). It is often difficult to clearly identify the luteal remnant macroscopically in follicular phase ovaries. Indeed, when identified, it may be more difficult to enucleate the corpus luteum at this time, as it is less delineated (Baird *et al.*, 1984). These factors aside, it is likely that menstrual corpora lutea could be collected successfully, and their inclusion would broaden these studies, and give additional information about normal luteolysis.

14.2.2 Tissue Fixation

It was clear from the studies reported in this thesis, that fixed tissue allowed better morphological clarity, and thus greater ease of interpretation, than frozen tissue sections. However, in most cases, some form of antigen retrieval was required to allow the detection of antigens by immunohistochemistry. Often, the immunohistochemical protocols required extensive refining to give reliable, and reproducible results. In addition, antigen retrieval was found to reduce the histological quality of the tissue. The fixative regimen used was quite harsh, and different fixatives were not explored. It is possible that optimal fixation was used in the protocol, but it remains possible that the tissues were over-fixed. Clearly, the fixation procedures could not be changed mid-experiments, but in the future there may be a role for an extended trial of different fixatives and different times of fixation. This may broaden the immunohistochemical uses of the tissues.

14.2.3 Alternative Sources of Tissue

Assuming there may be a small bias in the type of luteal tissue collected, is there any way that luteal tissue could be collected from younger women, with no gynaecological conditions that may be hormonally related? Unfortunately, there are very few alternative approaches to the collection of carefully dated human corpora lutea. An approach involving the collection of corpora lutea from normally cycling women having abdominal operations for non-gynaecological or non-uterine pathology would be impractical for several reasons. Firstly, such surgery would involve different surgeons and specialities, revised ethical approval, and a much more complex identification procedure. In addition, planned abdominal operations in young women of reproductive age, with regular cycles are uncommon. Emergency surgery, such as that required in acute appendicitis, would eliminate urinary dating, which is one of the strengths of the current study.

It is possible that the rigorous screening used in hysterectomy patients would not be as robust in general surgery patients, as gynaecological information is often missing from the notes.

Other major gynaecological operations are not suitable. Laparotomy for reversal of sterilisation or tubal surgery are much more infrequent procedures, and it is known that adhesions after ovarian surgery (Dabirashrafi *et al.*, 1991; Naether and Fischer, 1993; Duncan *et al.*, 1994) may impair fertility (Weinstein and Polishuk, 1975; Toaff *et al.*, 1976). However, there is another potential gynaecological source. The group of patients that would form the best source of normal human corpora lutea are those patients undergoing elective sterilisation. They are all of proven fertility, and thus proven normal luteal function, they tend to be younger (Wilcox *et al.*, 1991) than women having hysterectomy, and the theoretical risk of peri-ovarian adhesions affecting fertility would not apply. In addition, this is a common operation, potential subjects are plentiful, and operation dates are more flexible.

Over the period of the study, when 598 sets of pre-operative hysterectomy case notes were examined, over 2000 female sterilisation operations were performed in the Royal Infirmary of Edinburgh. Human luteal tissue, collected at sterilisation, has been used by other groups (Hagstrom *et al.*, 1996). There are, however, several considerations which makes these patients unsuitable at present. Firstly, the majority of women undergoing sterilisation are using hormonal rather than barrier forms of contraception, but more importantly, the vast majority of these operations are done laparoscopically (Penney *et al.*, 1997). Laparoscopic surgical skills are increasing, and laparoscopic ovarian cystectomies, and treatment of ectopic pregnancies (Alper *et al.*, 1992), are common-place. Although laparoscopic luteectomy or luteal biopsy is certainly possible, it is difficult, and not common practice.

At present, it is impractical, and probably unethical, to perform laparoscopic luteal biopsy. The operation time would be significantly increased. This, coupled with the potential of the highly vascular corpus luteum (Ford *et al.*, 1982) to bleed, (Raziel *et al.*, 1993) and the potential requirement for an open procedure (Raziel *et al.*, 1993), would increase the morbidity of the operation unacceptably. In addition, advanced laparoscopic surgery is a skill with a steep learning curve, and not all surgeons are competent at performing it. Therefore, while it should be remembered that sterilisation patients are a potential source of luteal tissue, with

present skills and equipment, it is not practical or safe to consider collecting such tissue. Whether things may change in the future is uncertain. It is therefore likely that there is little we can do to improve the numbers, or types, of corpora lutea collected.

14.2.4 Alternative Treatments in the Human Model

In the future, the techniques to develop a model for luteolysis in the human may be available. More and more data on the clinical use of $GnRH_{ant}$ in humans is being collected. These drugs are currently being used in assisted conception research programmes. It is possible that $GnRH_{ant}$ could be used safely to induce luteolysis in the human, in order to investigate co-ordinated luteolysis and allow the discontinuation of the marmoset model.

One of the fortuitous, but unexpected, findings of this thesis is that it may be possible to induce co-ordinated luteolysis using hCG withdrawal. One sample was collected three days after the final hCG injection, during a 'rescue' programme. Although this treatment excluded the sample from analysis, MMP expression was investigated in it by gelatine zymography (**Chapter 12**; Duncan *et al.*, 1998b). Although, only this one sample was available, it appeared that the increase in MMP activity during the late-luteal phase was accentuated. This model has never been used before, but it should be investigated as a potential model of co-ordinated luteolysis, both morphologically, functionally and structurally.

Other stages of luteal development are of interest, once an understanding of the luteal changes during the functional luteal phase have been investigated. Corpora lutea from ectopic pregnancies have been used to imply the luteal changes of early pregnancies. Although this can be criticised, an investigation of these corpora lutea may give information about the failing corpus luteum or the corpus luteum of later pregnancy. Whether used to improve the investigation, or merely to discredit luteal tissue from ectopic pregnancy, collection of this tissue should be considered in combination with the other tissues available. Although ectopic pregnancies are common (Li *et al.*, 1991b; Duncan *et al.*, 1995), the increasing use of laparoscopic surgical treatment may make the collection of tissues more infrequent, and difficult than it was previously (McNeilly *et al.*, 1980; Bramley *et al.*, 1987).

Another stage of the luteal lifespan which has not been well investigated is the corpus luteum after the luteo-placental shift. Interestingly, the luteal remnant can sometimes be identified on one of the ovaries at caesarean section at term, and may even be functional (Emmi *et al.*, 1991; Sherwood, 1994). A collection of these corpora lutea may provide valuable information about the corpus luteum in pregnancy (Zeleznik and Fairchild Benyo, 1994).

14.3 The Findings of this Thesis

This thesis has reported several novel findings. The LH receptor and other elements of the steroidogenic pathway are maintained, and not down-regulated, during maternal recognition of pregnancy in women (**Chapter 5**; Duncan *et al.*, 1996a). It has shown that induced luteolysis involves a rapid loss of the LH receptor and other elements of the steroidogenic pathway (**Chapter 6**; Duncan *et al.*, 1998a). Luteolysis is therefore clearly associated with a loss of the steroidogenic pathway. However, the data reported here support the hypothesis that progesterone levels decline before the changes in the steroidogenic pathway can be detected (**Chapter 7**). It appears that the loss of the steroidogenic pathway may be a consequence of the falling progesterone levels rather than the cause. However, once progesterone levels fall, the reduction in the steroidogenic pathway is likely to augment this decline.

Remodelling of the corpus luteum is of major importance to its structure and function. TIMP-1 is a major product of the human (**Chapter 10**; Duncan *et al.*, 1996b) and non-human primate (**Chapter 11**; Duncan *et al.*, 1996c) corpus luteum. It appears that it may have a role in preventing MMP action, because expression of TIMP-1 declines during induced luteolysis (Duncan *et al.*, 1996c). However, this effect is not seen in functional corpora lutea during natural luteolysis in women (Duncan *et al.*, 1996b). Here it seems likely that remodelling is controlled by an alteration in MMP expression (**Chapter 12**; Duncan *et al.*, 1998b). MMPs can work in an environment containing large amounts of TIMPs as they have different cellular localisations (Duncan *et al.*, 1998b). The MMPs seem to effect remodelling from the outside of the gland in. It is not sure why TIMP-1 is produced in large amounts by the corpus luteum; it is possible that it works as a facilitator of steroidogenesis (Duncan *et al.*, 1996c). MMP expression and action are inhibited during maternal recognition of pregnancy, by the action of

hCG. This effect is directly on MMP rather than TIMP expression (Duncan *et al.*, 1996b; Duncan *et al.*, 1998b).

Luteolysis in the functional luteal phase is associated with an influx of macrophages (**Chapter 13**; Duncan *et al.*, 1998c). This is inhibited by hCG during maternal recognition of pregnancy (Duncan *et al.*, 1998c). It is interesting that hCG acts through the LH receptor. The distribution of LH receptor expression indicates that it is unlikely that macrophages, or the cells involved in MMP production, express these receptors (Duncan *et al.*, 1996a). LH/hCG receptors are found on steroidogenic cells. It is therefore likely that steroidogenic cell products are involved in controlling macrophage influx and MMP expression. The nature of these products are not clear. One of the potential products is progesterone itself. Progesterone receptors can be found on the stroma and steroidogenic cells of the corpus luteum (**Chapter 8**), indicating that progesterone may have an autocrine role in the corpus luteum, and that it may be involved in controlling its own production. However, although the progesterone receptors present are those associated with classical hormone action, their role is unclear as they are present in small amounts, and at constant levels, despite changing ligand concentrations.

14.4 Speculative Paradigms

How can these findings be developed into a model of how the human corpus luteum works? The corpus luteum is a dynamic gland and models, therefore, have to take this into account. However, for the purpose of paradigm development, the corpus luteum can thought of in four main stages: the mid-luteal phase with maximal progesterone production; the late-luteal phase when progesterone production declines; the end of the late-luteal phase when progesterone production ceases; and during luteal 'rescue' when progesterone production is maintained. The following models are speculative, but encompass the general understanding of primate luteal function (Behrman *et al.*, 1993; Zeleznik and Fairchild Benyo), combined with the new information presented in this thesis.

14.4.1 The Mid-Luteal Corpus Luteum

The mid-luteal corpus luteum is illustrated diagramatically in Fig. 14.1. A small amount of LH is required to stimulate steroidogenesis. LH binds to its receptor

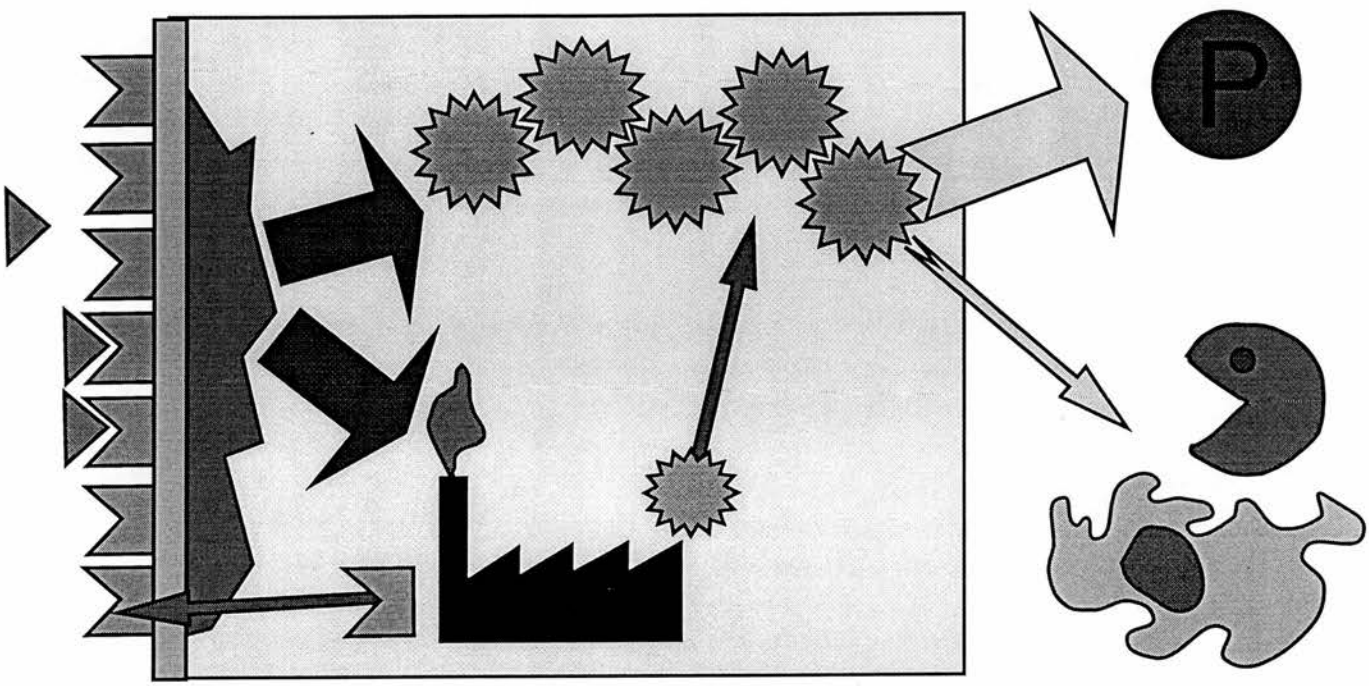

Figure 14.1

The mid-luteal corpus luteum

Speculative cartoon of the mid-luteal corpus luteum. LH binds to its receptor on the luteal cell membrane. This stimulates second messenger cascades. These messengers stimulate the production of progesterone and alter factors that result in a relative inhibition of macrophage influx, and MMP expression. The second messengers also facilitate continued synthesis of LH receptors, and other elements of the steroidogenic pathway.

and activates second messenger pathways. These molecules, particularly cAMP, stimulate the steroidogenic enzymes to synthesis progesterone. They also cause the continued synthesis of LH receptors and other elements of the steroidogenic pathway. The luteal cells are maximally functional, producing progesterone, and altering the synthesis of other products, that inhibit MMP expression and macrophage influx. The luteal cells also produce TIMP-1 which stabilises the local ECM, promotes interaction of the luteal cell with its local matrix, and neighbours, and inhibits tissue remodelling by MMPs. The functionality of the steroidogenic cell is improved by these actions, and progesterone synthesis is facilitated.

14.4.2 The Late-Luteal Phase

The late-luteal corpus luteum is illustrated in Fig. 14.2. The small amount of LH binds to the LH receptors and activates the second messenger pathways. However the same amount of LH stimulates a reduced amount of second messenger molecules. There is evidence of changes in the enzyme, adenylyl cyclase (Eyster *et al.*, 1985; Rojas *et al.*, 1989), and a functional uncoupling of the LH receptor (Segaloff and Ascoli, 1993). This thesis speculates that the LH receptor increasingly uncouples from the second messenger pathways. Several authors have suggested that the corpus luteum, when formed, is programmed to die (Behrman *et al.*, 1993; Zeleznik and Hillier, 1996). It is possible that the increasing uncoupling of the LH receptor is a function of the maturation of the corpus luteum throughout the luteal phase.

The resulting reduced concentrations of second messengers, notably cAMP, stimulates less progesterone output. In addition, it is postulated that less LH receptors and other elements of the steroidogenic pathway are synthesised. This will reduce the components of, as well as the stimulation of, the steroidogenic pathway. One of the effects of this is to start a cycle of reduced generation of cAMP, reduced progesterone synthesis, reduced synthesis of the components of the steroidogenic pathway, and therefore reduced ability to respond to cAMP. The result of this cycle, is that progesterone concentrations increasingly decline and the corpus luteum heads inexorably towards its luteolytic fate. As well as the declining progesterone, the production of other luteal cell products change. It is not known whether it is the reduced production of luteal products, or the increased production of some factors, during luteolysis, that effects macrophage influx.

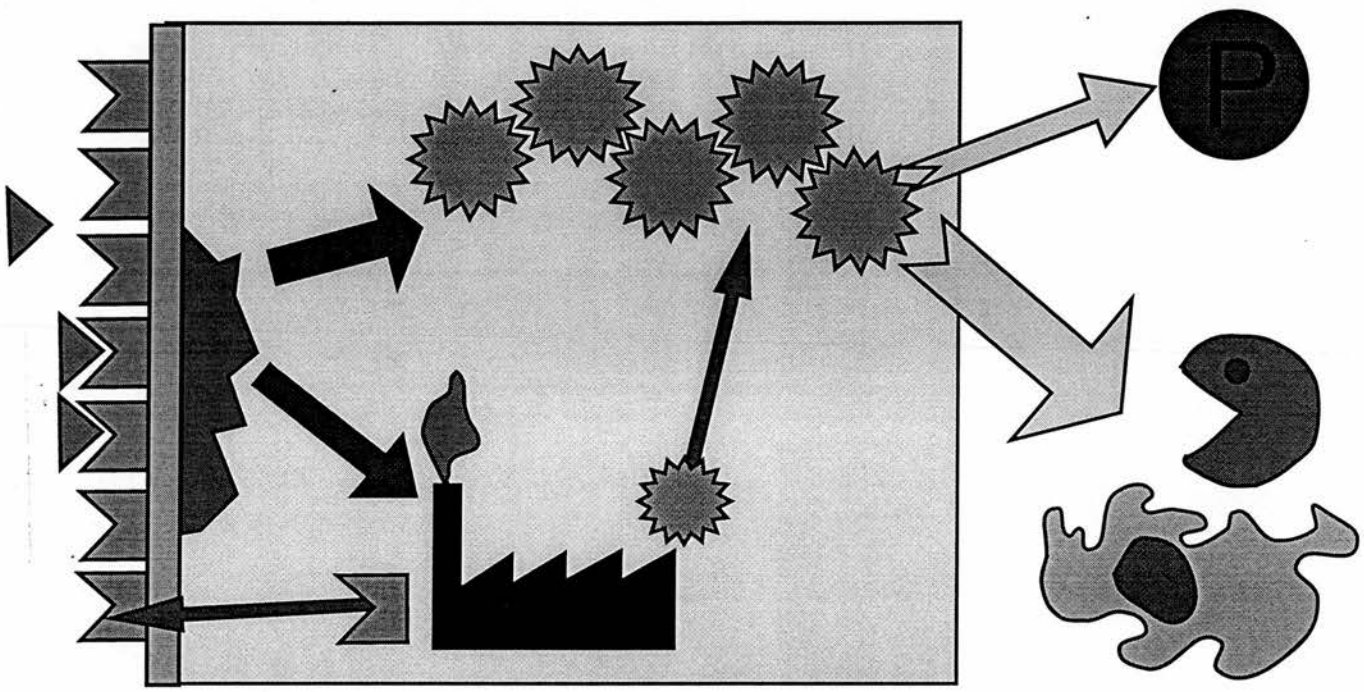

Figure 14.2

The late-luteal corpus luteum

Speculative cartoon of the late-luteal corpus luteum. Here, LH binds to its receptor on the luteal cell membrane but, because of increasing receptor uncoupling, less second messenger molecules are produced. This results in less progesterone being synthesised, and less replenishment of LH receptors and other elements of the steroidogenic pathway. This further reduces progesterone output. Factors synthesised by the corpus luteum are altered to favour increasing macrophage influx and MMP expression and activity.

However, there is an associated influx of macrophages and induction of MMP expression and activity.

The role of the macrophages is not entirely clear. Their products may inhibit steroidogenesis further (Brännström and Norman, 1993). It is also likely that macrophage products are involved in increasing the remodelling of the ECM (Hurwitz *et al.*, 1993; Hulboy *et al.*, 1997). There is clearly some ECM remodelling and increased activity of MMPs at this stage. This is likely to be secondary to a change in MMP/TIMP ratios. Cells which lose their contact with the ECM are more likely to die by apoptosis (Pullan *et al.*, 1996). It is possible that cell death is instigated by this remodelling. It is also possible that the cells, with their failing progesterone production, are producing more death factors and less survival factors (Fraser *et al.*, 1995b; Rodger *et al.*, 1995; Spencer *et al.*, 1996; Rodger *et al.*, 1998). Macrophages can begin the process of phagocytosis of cellular and tissue debris (Paavola, 1979).

14.4.3 The End of the Late-Luteal Phase

At the end of the functional luteal phase, there is little response to the ambient LH. This is because there is little second messenger stimulation from the LH receptor, and the components of the steroidogenic pathway are increasingly depleted (Fig. 4.3). Studies have shown that menstrual primate corpora lutea do not contain LH receptors (Ravindranath *et al.*, 1992a; Nishimori *et al.*, 1995), or other elements of the steroidogenic pathway (Doody *et al.*, 1990; Bassett *et al.*, 1991). In addition, the speculative factors inhibiting macrophage influx or MMP expression are also reduced. Macrophages are present in large amounts in this stage. They are likely to produce MMPs or stimulate MMP expression. The MMP/TIMP ratio increases, as MMPs are increasingly expressed (Duncan *et al.*, 1998b), and it is likely that, with the demise of steroidogenic cells, TIMP-1 levels decline (Duncan *et al.*, 1996c). The ECM is increasingly being remodelled, primarily from the outside of the gland inwards, but also in small islands within the steroidogenic cell layer (Duncan *et al.*, 1998b).

At this stage, luteal cells begin to die by apoptosis and the gland shrinks. The stimulus to apoptotic cell death is not clear, but it is likely to involve several factors. The loss of cell contact with the ECM (Pullan *et al.*, 1996; Aston *et al.*, 1996b) is likely to play some role. In addition, proto-oncogene expression can be detected in the corpus luteum (Rodger *et al.*, 1995; Fraser *et al.*, 1995b; Rodger *et*

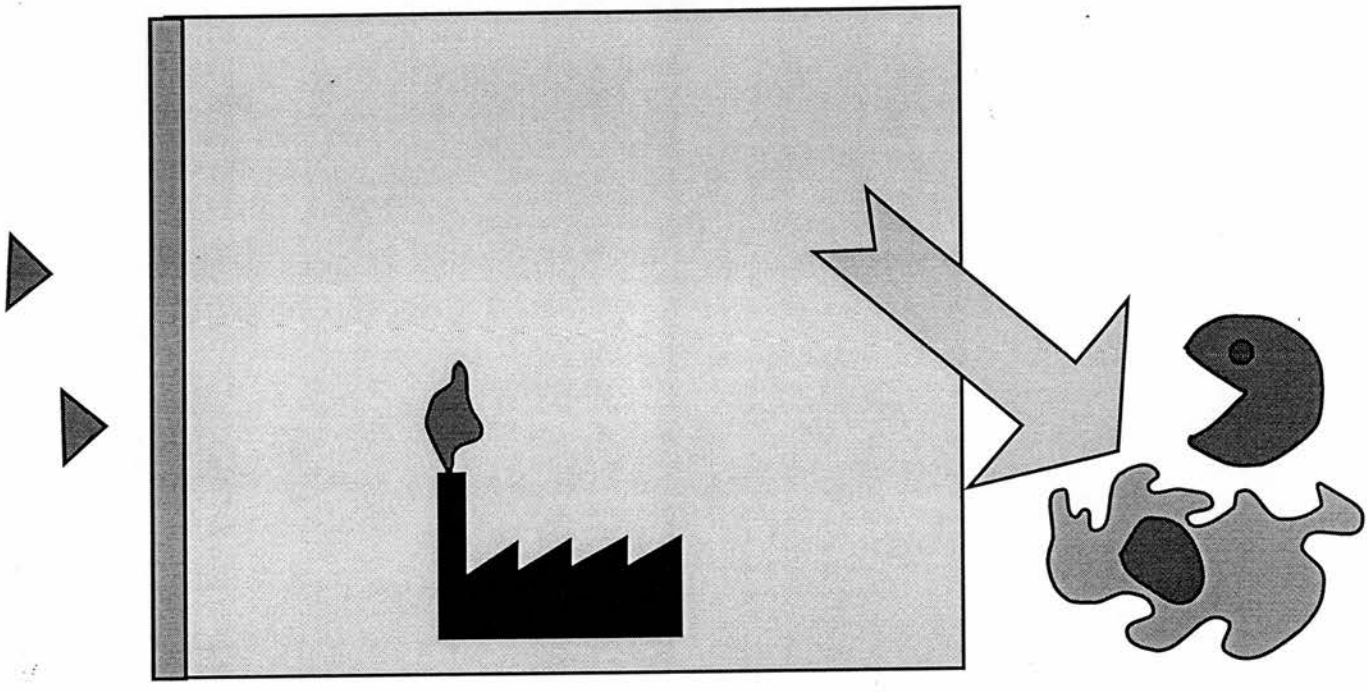

Figure 14.3

The corpus luteum at the end of the luteal phase

Speculative cartoon of the corpus luteum at the end of the luteal phase. There are few LH receptors and other elements of the steroidogenic pathway. Progesterone synthesis has therefore ceased. The balance of cell products now favours the marked influx of macrophages and the expression and activity of MMPs.

al., 1998). Although significant differences in proto-oncogene expression, throughout the luteal phase, have not been consistently reported. It is possible that, like in the atretic follicle (Tilly, 1996; Spencer *et al.*, 1996; Gougeon, 1996), an alteration in the expression of cellular survival and death genes (Tilly, 1996) is involved in initiating luteal cell death. Clearly the macrophage is involved in the phagocytosis of apoptotic bodies and the clearance of cellular debris. It is also possible that endothelial cells, as well as steroidogenic cells, die (Fraser *et al.*, 1995c; Modlich *et al.*, 1996). This would block local capillary blood flow (Modlich *et al.*, 1996), and reduce the blood supply to the corpus luteum. The net effect of these processes is a reduction in the size of the gland, increased tissue remodelling, and a marked reduction in its vascularity.

14.4.4 Luteal 'Rescue'

The cellular events surrounding luteal 'rescue' are illustrated in cartoon form in Fig. 14.4. The reduced stimulation of the second messenger system, possibly second to functional uncoupling of the LH receptor, is still present. As the corpus luteum matures, as postulated above, this uncoupling increases. However, there are increasing amounts of hCG in the circulation (Lenton and Woodward, 1988). This hCG acts through the LH receptor. As the primate LH receptor does not down-regulate in the presence of an excess of its ligand (Duncan *et al.*, 1996a), this results in an increased stimulus to the second messenger pathways. The increasingly high ligand levels, in the presence of increasingly uncoupled receptors, maintains a similar amount of second messenger molecules. This maintains the production of progesterone and the synthesis of each element of the steroidogenic pathway.

As well as maintaining the production of progesterone, the factors inhibiting MMP expression and macrophage influx are maintained. This maintains, or reduces, the MMP/TIMP ratio. The ECM is stabilised, and in the presence of a stable ECM, and a potential excess of survival factors, apoptosis of the luteal cells is inhibited. There is no proliferation of the endothelial cells during luteal 'rescue' (Christenson and Stouffer, 1996a; Rodger *et al.*, 1997), but luteal blood flow is clearly maintained (Glock and Brumsted, 1995; Kupesic and Kurjak, 1997). The increasing LH receptor uncoupling requires logarithmically increasing hCG to maintain adequate adenylyl cyclase stimulation, and progesterone production. However, the life of the corpus luteum can only be prolonged in the short term,

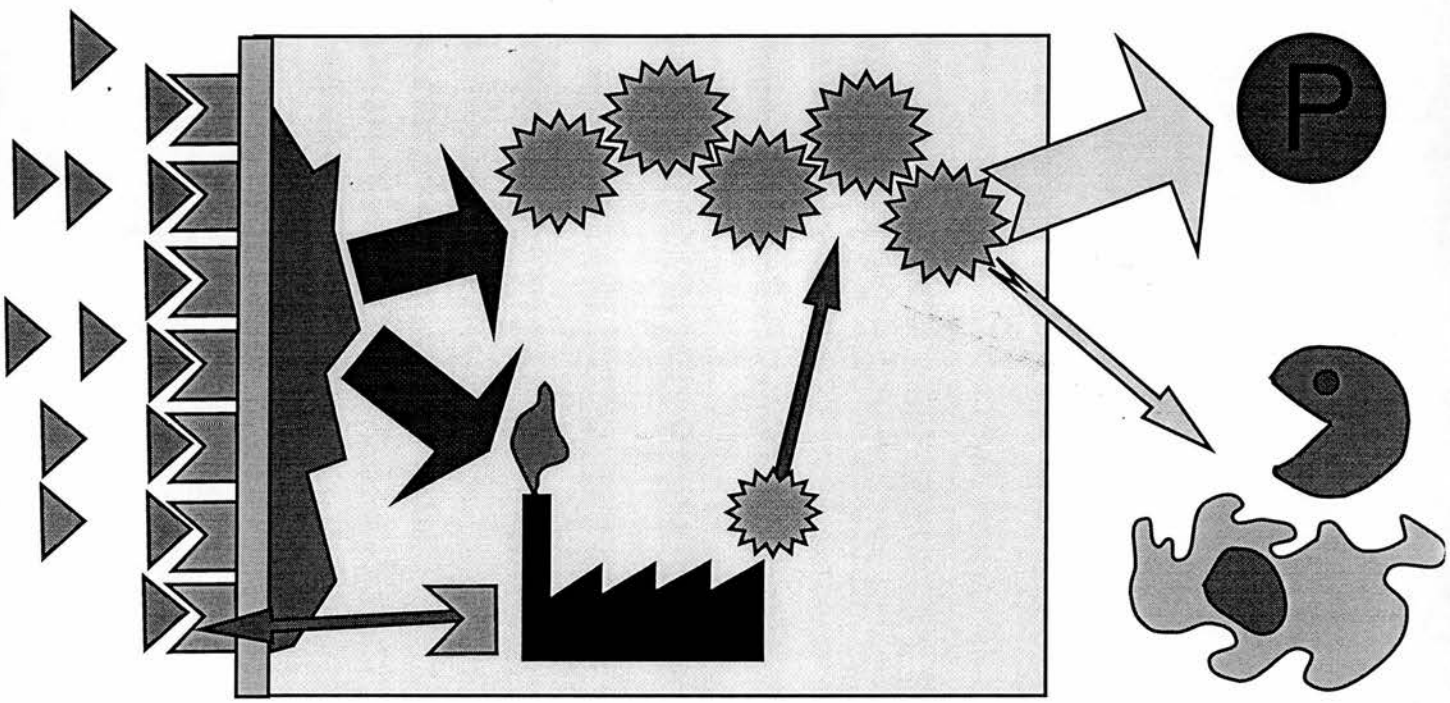

Figure 14.4

Luteal 'rescue' during maternal recognition of pregnancy

Speculative cartoon of the cellular mechanisms in luteal cells at the time of luteal 'rescue' by hCG, during maternal recognition of pregnancy. The presence of large amounts of hCG causes increased stimulation of the LH receptors on the luteal cell membranes. Although, the LH receptor is increasingly uncoupled, this increased receptor stimulation maintains the levels of intracellular second messengers. These molecules maintain steroidogenesis and stimulate the continued synthesis of LH receptors and other elements of the steroidogenic pathway. The continued function of the luteal cells, changes the balance of products to prevent macrophage influx and MMP expression.

and by the time of the luteo-placental shift, the increasing synthesis of hCG cannot be maintained and, as the corpus luteum is now relatively unreactive to hCG, regression begins.

14.5 Strategy for Further Investigation

It is not known how accurate the above paradigms are. However, it is likely that there is some degree of relevance to how the human corpus luteum works. What the above paradigms do achieve, is the development of hypotheses, that can be validated or refuted by further study. The corpus luteum has been likened to an unfinished jigsaw. Many of the pieces are still missing. However, the development of the speculative pathways, outlined above, has highlighted several key areas that need to be defined and explored. The following section focuses on some of these areas, and begins the search for some crucial missing pieces.

14.5.1 Luteal LH/hCG Action

When considering the speculative paradigms above, there are several assumptions that are made. The first premise is the functional uncoupling of the LH receptor as the corpus luteum matures. This is fundamental to the theories about the control of the primate corpus luteum presented in this thesis. Such uncoupling has previously been suggested by *in vitro* studies of the LH receptor (Ezra and Salomon, 1980; Segaloff and Ascoli, 1993). However, previous strategy for the study of the LH receptor, has involved the study of these receptors in mouse cell lines, or in rat tissues (Segaloff and Ascoli, 1993). This approach has its problems. It is clear that there are fundamental differences in the behaviour of the LH receptor in rodent and primate luteal tissues, particularly with reference to down-regulation (Caldwell *et al.*, 1980; Peegel *et al.*, 1994; Duncan *et al.*, 1996a). Clearly studies of the coupling of the LH receptor are required in human cells expressing the human LH receptor.

Studies on the LH receptor, and the coupled enzymatic complex, may be possible on luteinised granulosa cells (Soto *et al.*, 1984; Sjögren *et al.*, 1991; López Bernal *et al.*, 1995; Takao *et al.*, 1997). Indeed, this may be the best way to investigate the human LH receptor in the first instance. However, one of the major questions to be answered involves the nature of the differences between the behaviour of the

rat LH receptor and that of the primate. Why does the human LH receptor not down-regulate? It is difficult to envisage how this question can be addressed. A comparison of human and rat LH receptor gene response elements, and functional studies involving transient gene expression of these receptors in rat and human cells, may give additional information.

An investigation of LH receptors, and their coupled intercellular responses, requires careful *in vitro* studies of luteal cells. The response on luteal cells to LH/hCG *in vitro*, at different stages of the luteal phase, has been investigated in the past (Stouffer *et al.*, 1977). However, these experiments should be repeated with the newer molecular, and cell imaging, techniques now available. Clearly, a careful study of the numbers and availability of LH receptors (Cameron and Stouffer, 1982; Ottobre *et al.*, 1984; Bramley *et al.*, 1987; Segaloff and Ascoli, 1993), coupled with the ability of ligand to stimulate second messenger molecules, *in vitro*, would be of great interest. There is, therefore, a role for continuing *in vitro* studies of luteal cell function.

It is possible to investigate luteal steroidogenic cells *in vitro*. In general, individual luteal cells are obtained by enzymatic digestion of corpora lutea (Lei *et al.*, 1991; Brannian *et al.*, 1993). However, enzymatic digestion can effect the integrity of membrane proteins, including the LH receptor. It has therefore been suggested that mechanical separation and elutriation of individual cells may be a more useful technique to obtain intact luteal cells (McLean *et al.*, 1992). It has been noted, however, that other cells, particularly white cells, that contaminate cultures (Spanel-Borowski and Ricken, 1997), can affect steroidogenesis (Kirsch *et al.*, 1981; Kirsch *et al.*, 1983; Halme *et al.*, 1985). This may be a problem in the assessment of primary cultures from different stages of the luteal phase, as the immune cell content of corpora lutea changes throughout the luteal phase (Wang LJ *et al.*, 1992; Brännström *et al.*, 1994a; Best *et al.*, 1996; Duncan *et al.*, 1998c). In addition, primary cell culture involves a breakdown in intercellular communications, and an alteration in the cellular composition, and relationships, that may be of fundamental importance to normal luteal function. One solution, to this problem, may be the use of thin luteal slices (Girsh *et al.*, 1996; Hagstrom *et al.*, 1996; Fairchild Benyo and Zeleznik, 1997). This technique has the potential to be more accurate than single cell culture, in that cells remain intimately associated with their natural neighbours. However, the effects of the inevitable tissue damage, and cytokine release, during slicing, remain uncharacterised.

It is possible that there is a change in the nature of the LH receptor itself that effects its ability to couple to G-proteins. The LH receptor is known to undergo alternate splicing, and several splice variants have been detected. LH receptor splice variants have been reported in rodents (Aatsinki *et al.*, 1992), sheep (Bacich *et al.*, 1995), and pigs (VuHai-LuuThi *et al.*, 1992). Indeed, it is thought that the full length receptor is only a minority of the LH receptor mRNA synthesised (Bacich *et al.*, 1995). These splice variants tend to lack varying amounts of the mRNA which codes for the transmembrane domains (Themmen *et al.*, 1994). Indeed, once translated, different splice variants appear to have different functional characteristics. It is likely that changes in the levels of different splice variants may effect the overall functionality of the LH receptor. It is not known, though, if these variants change throughout the luteal phase. However, it has recently been shown that both marmoset testicular, and human luteal, tissues do express LH receptor splice variants (Zhang *et al.*, 1997; Minegishi *et al.*, 1997). Clearly studies on the functionality, distribution, and ontogeny, of these splice variants in the human corpus luteum are required.

There is, therefore, still a place for the continued study of LH/hCG receptor in human corpora lutea. Clearly though, a robust model of LH receptor expression and coupling to second messenger pathways, is required. This, for practical purposes, is likely to be a cell culture based system. Techniques for the estimation of cAMP formation *in vitro* have been developed (López Bernal *et al.*, 1995). In addition, calcium imaging can be used to look at intracellular effects of receptor action in hormone sensitive tissues (Anderson *et al.*, 1996). It is therefore possible to study the stimulation of second messengers in response to LH binding. These experiments should, in addition, be combined with an investigation of the steroidogenic effects of membrane permeable formulations of cAMP, *in vitro* (Jordan, 1981; Soto *et al.*, 1984). This has the potential to dissect the contributions of receptor uncoupling to adenylyl cyclase, and the down-stream actions of cAMP, during functional luteolysis. It is likely that we have not heard the last about the LH receptor of the primate corpus luteum.

14.5.2 Paracrine Molecules

It is not clear what factors control the local effects of hCG in the corpus luteum, postulated in the paradigms described above. There are many different potential paracrine molecules in the corpus luteum (Behrman *et al.*, 1993; Nappi *et al.*,

1994; Zeleznik and Fairchild Benyo, 1994). Clearly, each potential regulatory molecule needs to be detected and localised in primate corpora lutea in the first instance. Once identified and characterised, the effects of these molecules, and the factors controlling their expression, need to be investigated. It is only then that the contribution of each individual molecule can be mapped. As there are any number of potential regulatory molecules, it is important to specifically target likely molecules for the initial detailed studies. One of the groups of molecules worthy of further investigation are the IGFs. This is because they have been shown to have a major role in folliculogenesis (Hillier, 1991; Jones and Clemmons, 1995), and they can be detected in the corpus luteum (Hernandez *et al.*, 1992; Johnson *et al.*, 1996).

A systematic investigation of the expression, localisation, and source, of the IGFs in the human corpus luteum throughout the luteal phase and after luteal 'rescue' is indicated. In addition, the cellular localisation of IGF receptors, in the same circumstances, would be of interest. It is now clear that the actions of IGFs can be modified by IGFBPs (Jones and Clemmons, 1995). It is also clear that the ECM can form a repository for these molecules (Jones and Clemmons, 1995; Hulboy *et al.*, 1997), and that any agent which modifies the ECM can alter local IGF bioavailability. One of the most interesting recent developments is the localisation of IGFBP-3 to the vascular endothelial cells of the corpus luteum (Fraser *et al.*, 1997). The role of IGFBP-3, and its expression throughout the luteal phase, are clearly an important area for study.

There is increasing interest in the role of glucocorticoids in ovarian function (Daly *et al.*, 1984; Wang *et al.*, 1993; Waddell *et al.*, 1996; López Diaz and Bosu, 1997). A variety of sex steroid receptors can be localised to the corpus luteum (Horie *et al.*, 1992; Suzuki *et al.*, 1994), and it appears that glucocorticoid receptors are no exceptions (Sugino *et al.*, 1997). In addition, corticotrophin releasing factor (CRF) may have a role in ovarian function (Apa *et al.*, 1995; Asakura *et al.*, 1997; Erden *et al.*, 1998). The role of adrenal steroids in the corpus luteum is not known. However, an interesting observation is the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) in rodent (Waddell *et al.*, 1996), and human corpora lutea (Ricketts *et al.*, 1998) This enzyme is involved in the conversion of cortisol to cortisone, and it serves to reduce a tissue's exposure to cortisol (Waddell *et al.*, 1996). There is some evidence that the expression of this enzyme changes during the luteal phase (Waddell *et al.*, 1996). A review of the

ability of the corpus luteum to synthesise local corticosteroids, respond to them, and alter their local bioavailability is therefore indicated. This investigation could start with a detailed analysis of the expression of nuclear glucocorticoid receptors, and 11β-HSD, in the corpus luteum, throughout the luteal phase and after luteal 'rescue' with exogenous hCG.

14.5.3 Inhibins and Activins

It is likely that inhibins and activins have major roles in the corpus luteum. It is only now that these potential roles are coming to light. Messenger RNA for the subunit building blocks of inhibin and activin are expressed in the primate corpus luteum (Fraser *et al.*, 1993; Roberts *et al.*, 1993). In addition, inhibin and activin proteins can be detected in the corpus luteum (Cuevas *et al.*, 1987; Smith *et al.*, 1991; Wada *et al.*, 1996), and in the peripheral circulation during the luteal phase of the ovarian cycle (Groome *et al.*, 1996; Muttukrishna *et al.*, 1996). It is likely that these proteins have an endocrine effect on pituitary FSH secretion (Burger and Igarashi, 1988; Fraser and Lunn, 1993). However, it is possible that they have paracrine effects in the corpus luteum. Inhibin and activin, in particular, have the potential to modulate luteal cell steroidogenesis (Hillier *et al.*, 1991a; Miro and Hillier, 1992; Brannian *et al.*, 1992). Indeed, expression of inhibin and activin changes during the luteal phase. Serum studies have suggested that inhibin A declines in the late-luteal phase (Groome *et al.*, 1996), at a time when activin A tends to increase (Muttukrishna *et al.*, 1996).

In vitro studies, and observational studies *in vivo*, have suggested that luteal cell inhibin, particularly the α-subunit, is stimulated by LH/hCG (Eramaa *et al.*, 1994; Illingworth *et al.*, 1996). It is therefore possible that gonadotrophin-stimulated expression of the α-subunit, promotes inhibin A expression in the functional corpus luteum, during the mid-luteal phase, and after luteal 'rescue' (Illingworth *et al.*, 1990; Illingworth *et al.*, 1996). It is also possible that a relative reduction in gonadotrophin action, in the late-luteal phase, reduces α-subunit expression (Fraser *et al.*, 1995a). This means that the relative concentrations of the β-subunits, which are less affected, increase. This serves to facilitate the synthesis of activin. Indeed, it appears that luteal activin A concentrations increase at the end of the luteal phase (Roberts *et al.*, 1993; Muttukrishna *et al.*, 1996). Activin A can promote cell death, and can directly inhibit steroidogenesis (Brannian *et al.*, 1992; Miro and Hillier, 1992; Nishihara *et al.*, 1995; Wang *et al.*, 1996).

This potential local role of activin in the corpus luteum during the luteolytic process, is highly speculative. Activin secretion at the end of the luteal phase may, solely, be an endocrine stimulus to pituitary FSH secretion in preparation for the early follicular phase (Baird and Smith, 1993). However, activin receptors have now been identified at a molecular level (Shinozaki *et al.*, 1992; Cameron *et al.*, 1994; Eramaa *et al.*, 1995), and they can be detected in luteinised granulosa cells (Eramaa *et al.*, 1995). There is, therefore, a role for the further investigation of activins and inhibins in the human corpus luteum throughout the luteal phase. Clearly classic endocrine and paracrine studies should be performed *in vivo*, when agonists and antagonists are available in large enough doses. However, in the first instance, a quantitative analysis of mRNA expression of each subunit throughout the luteal phase, and after luteal 'rescue', is indicated. In addition, molecular and functional studies to identify the expression, type and localisation of activin receptors in the human corpus luteum would also be of interest.

14.5.4 Prostaglandins

Further studies of the potential effector molecules, involved in primate luteolysis, are required. It is clear that prostaglandins, particularly $PGF_{2\alpha}$, have local roles in luteolysis in infraprimate species (Auletta and Flint, 1988; Niswender and Nett, 1994). In some primate species, notably the marmoset monkey, systemic $PGF_{2\alpha}$ is also luteolytic (Webley *et al.*, 1991; Fraser *et al.*, 1995b). In addition, there is evidence that prostaglandins may have a local role during luteolysis in the primate (Patwardhan and Lanthier, 1980; Auletta *et al.*, 1984a; Auletta *et al.*, 1984b; Auletta *et al.*, 1990; Zelinski-Wooten and Stouffer, 1990). It is certainly possible that primates and infraprimate species have similar local mediators of luteolysis. These mediators are likely to include $PGF_{2\alpha}$.

The $PGF_{2\alpha}$ receptor has now been cloned (Lake *et al.*, 1994; Graves *et al.*, 1995). Several studies have therefore reported the expression of these receptors in infraprimate corpora lutea (Rueda *et al.*, 1995b; Juengel *et al.*, 1996; Olofsson *et al.*, 1996; Mamluk *et al.*, 1998). Crude early binding studies reported $PGF_{2\alpha}$ receptors in human corpora lutea (Powell *et al.*, 1974; Rao *et al.*, 1977a), and recently receptor mRNA has been described in luteinised granulosa cells (Ristimaki *et al.*, 1997). It is not just $PGF_{2\alpha}$ that may be involved in controlling luteal function, other PGs have also been implicated (Zelinski-Wooten and Stouffer, 1990; Olofsson and Leung, 1994). It is now possible for an extended

molecular investigation of PG receptors in the human corpus luteum. The expression and localisation of PG receptors should be investigated throughout the luteal phase and after luteal 'rescue' with exogenous hCG in women, and throughout the luteal phase, and after induced luteolysis, in the marmoset.

The synthesis of prostaglandins, and how their synthesis is controlled, is also likely to be important. It is now possible to investigate the expression and the localisation of the major enzymes involved in PG synthesis and metabolism. Although the COX enzymes, and prostaglandin dehydrogenase (PGDH), have been studied in different systems, a systematic study of their expression in the human corpus luteum is required. The time is right for a revisitation of the effects of prostaglandins in the primate corpus luteum using current molecular technologies.

14.5.5 Progesterone

One of the most intriguing questions, about the control of the corpus luteum, remains unanswered. Does progesterone regulate its own synthesis, or the integrity of the gland, at a local level? It has been postulated that paracrine actions of progesterone are involved in controlling steroidogenesis (Rothchild, 1981; Duffy *et al.*, 1994; Rothchild, 1996). The initial technique used to investigate progesterone action in the corpus luteum utilised the progesterone antagonist, RU486. This approach had problems, as RU486 had direct effects on the secretion of luteotrophic gonadotrophins from the pituitary (Schaison *et al.*, 1985; Batista *et al.*, 1994). Newer, elegant techniques, using 3β-HSD inhibitors, are now being used to investigate the local effects of progesterone in the corpus luteum. Although these experiments are generating useful data, they also have associated problems, which makes full interpretation of the results difficult. Although the morphology of the corpus luteum is markedly altered (Duffy *et al.*, 1994), it appears that the corpus luteum continues to synthesise normal amounts of some hormones, notably relaxin (Duffy *et al.*, 1995; Duffy *et al.*, 1996). In addition, oestrogen synthesis was unexpectedly, and inexplicably, maintained during treatment with trilostane, in this experimental model (Duffy *et al.*, 1994). The results and conclusions of these studies remain controversial.

It may be that progesterone precursor molecules, accumulate in the corpus luteum after trilostane treatment. The build up of these steroids, using this method, may interfere with the structure and function of the corpus luteum *per se*. Although the

use of 3β-HSD inhibitors *in vivo*, has generated interesting results (Duffy *et al.*, 1994; Slayden *et al.*, 1994; Duffy and Stouffer, 1995; Duffy *et al.*, 1996), it is likely that other approaches are required. One approach may be to add back LH during treatment with RU486. An earlier study used hCG in addition to RU486, and found that RU486 had little effect on luteal function (Croxatto *et al.*, 1989). However, this was a crude and unphysiological approach. It may be that a more sophisticated control of pituitary LH after RU486 treatment is required.

One approach may be to use pulsatile LH, or GnRH, infusions to stimulate the corpus luteum, as used previously in the classic endocrinological experiments (Hutchison *et al.*, 1986; Zeleznik and Fairchild Benyo, 1994). This would allow physiological LH concentrations to be present in the presence of RU486 or excess additional exogenous progesterone. The effect of this regimen on the corpus luteum should be studied both functionally and structurally. Parameters for study should include secretory products of the corpus luteum. If the progesterone concentrations cannot be adequately assessed, other hormones such as relaxin (Sherwood, 1994) and inhibin A (Groome *et al.*, 1996), should be investigated. In addition, corpora lutea should be collected for structural and functional investigation *in vitro*. Morphological indicators of luteal function, such as LH receptor and steroidogenic enzyme expression, MMP expression, and macrophage influx should be studied. It is of interest that rodent models cannot be used for such studies as, unlike primates, they do not express luteal progesterone receptors (Parke-Sarge *et al.*, 1995).

There is continuing debate about specific non-genomic progesterone receptors in the corpus luteum (Rae *et al.*, 1998). Non-genomic actions of progesterone may be secondary to non-specific membrane effects, or specific membrane associated receptors. It is not known whether these receptors exist as distinct entities, but there is quite convincing evidence, from sperm studies, that progesterone has actions that cannot be explained by the presence of classical nuclear progesterone receptors (Luconi *et al.*, 1998; Revelli *et al.*, 1998). Indeed, there is an increasing body of evidence for membrane-associated progesterone binding sites, in infraprimate corpora lutea (Bramley and Menzies, 1988; Bramley and Menzies, 1994; Rae *et al.*, 1998). The corpus luteum may, therefore, prove a source of tissue in the molecular search for the non-genomic progesterone receptor. The local effects of progesterone, at genomic and membrane levels, on cellular

function in the corpus luteum are not fully understood. However, they are likely to be important, and suitable for further study.

14.5.6 Cellular Connections and Fine Structure

It is increasingly clear that cellular connections, with each other and the ECM, are very important for normal tissue function. There is recent evidence about the expression of adhesion molecules, integrins, connexins and gap junction molecules in the corpus luteum (Okuma *et al.*, 1996; Khan-Dawood *et al.*, 1996b; Khan-Dawood *et al.*, 1996c; Simon *et al.*, 1997; Honda *et al.*, 1997). One of the potential ways that MMPs may effect tissue remodelling and affect luteal function, is by catalysing the alteration, or destruction, of these molecules. Indeed, if these molecules are of primary importance in luteal function, one of the functions of TIMPs may be to stabilise, and maintain them. Clearly, a systematic investigation of these molecules, throughout the luteal phase, and after luteal 'rescue' in the primate, would be timely.

One of the most powerful tools in the analysis of dynamic tissue processes, is snap shot studies at the morphological level. Such studies have clarified the tissue processes surrounding ovulation (Pederson, 1951; Tsafriri and Dekel, 1994; Espey and Lipner, 1994), and increased our basic understanding of how ovulation occurs (Espey, 1994). There have been careful studies on the histological structure (Corner, 1956), and fine structure (Gillim *et al.*, 1969), of the human corpus luteum throughout the luteal phase. However, these studies are now several decades old. There is clearly a role to reassess the morphology and ultrastructure of the corpus luteum, as additional information could now be collected. This is because: there have been marked improvements in the quality of microscopes, and higher power light magnification; the corpus luteum could be assessed in light of current knowledge; clear endocrinological dating of the corpus luteum is possible; additional immunohistochemical techniques are now available for cellular identification and clarification; and it is now possible to use the luteal 'rescue' model to investigate the changes of early pregnancy.

A systematic morphological study of the human corpus luteum would be of great interest in the present climate. Indeed, there has been a move towards more morphological studies of the corpus luteum (Kim-Björklund *et al.*, 1991a; Kim-Björklund *et al.*, 1991b; Retamales *et al.*, 1994; Khan-Dawood *et al.*, 1996a; Torii *et al.*, 1996). A simple re-analysis of the morphological changes in luteolysis and

luteal 'rescue' may give pointers to future areas of study. In addition, it is easier to understand the distribution of mRNA and protein species in the corpus luteum, if a clear description of its detailed morphology, and dynamic morphological and temporal changes, is available. There is still a role for simple morphological analysis of the corpus luteum.

14.5.7 Tissue Remodelling

This thesis has provided the first evidence about MMP and TIMP expression in the primate corpus luteum. There are several potential areas of study to expand these observations. The first area, is an investigation of the nature of the cells expressing MMP-9 in the human corpus luteum. MMP-9 is expressed is individual cells in the granulosa-lutein cell layer. Good antibodies for MMP-9 are now available (Nikkari *et al.*, 1996). It should now be possible to investigate the cellular localisation of MMP-9 by immunohistochemistry, in sections of human corpus luteum. Indeed, it is possible that this immunohistochemistry could be performed on fixed sections, with improved morphological definition.

If better morphological definition is not sufficient to allow the identification of these cells, then dual staining immunohistochemistry (Rodger *et al.*, 1995), with white cell, and steroidogenic cell markers may help. Now techniques exist for a combination of isotopic mRNA *in situ* hybridisation with immunohistochemistry (Millar *et al.*, 1995), and these techniques may be valuable assets in the investigation of the cellular localisation of MMP-9. However, one of the confusing aspects of MMP localisation in the corpus luteum, is whether granulosa-lutein cells themselves are able to synthesise and secrete MMPs. It is certainly thought they can in culture (Puistola *et al.*, 1996a; Aston *et al.*, 1996a). However, in tissue sections, investigation of MMP expression by mRNA *in situ* hybridisation, revealed only minimal levels of expression in these cell types (Duncan *et al.*, 1998b). Whereas it is likely that luteinised granulosa cells, deprived of normal cellular connections, in culture, themselves secrete MMPs, it is possible that the results are influenced by white cell contamination of these cultures (Spanel-Borowski and Ricken, 1997). Immunocytochemistry on cytospin granulosa cell cultures, using white cell, and steroidogenic cell markers, may help identify the cellular source of MMPs in these experiments.

Another interesting area highlighted is the role of TIMP-1 as a facilitator of steroidogenesis. Further work requires to be done on the expression of TIMP-1 in

non-luteal steroidogenic tissues. In addition, its direct steroidogenic effects in culture (Boujrad *et al.*, 1995), need to be confirmed in different culture conditions, in various endocrine systems, and by other groups. The mechanism of the steroidogenic effects of TIMP-1, in culture, requires dissection, especially in light of the finding that bovine TIMP-1 has sequence homology with StAR (Hartung *et al.*, 1995). The steroidogenic effect of TIMP-1 is thought to occur in combination with procathepsin-L (Boujrad *et al.*, 1995). The expression and localisation of procathepsin-L in the primate corpus luteum, and in other TIMP-expressing tissues, has not yet been studied. This needs to be documented.

There are at least 17 forms of MMP (Hulboy *et al.*, 1997). This thesis has investigated the more common ones. It remains possible that other MMPs have a major role in the function and structure of the corpus luteum, throughout the luteal phase and during maternal recognition of pregnancy. Further studies on the other MMPs, particularly matrilysin (MMP-7) and the stromelysins 1,2 and 3 (MMP-3, MMP-10, MMP-11), are required. They appear to have roles in menstruation (Salamonsen and Woolley, 1996; Hulboy *et al.*, 1997), a process with many parallels with luteolysis. Serine proteinases have been implicated in the local activation of MMPs (Hulboy *et al.*, 1997). Indeed, plasminogen activator has been identified in the ruminant and rat corpus luteum (Liu *et al.*, 1996; Smith *et al.*, 1997). Clearly, information on its expression in the human corpus luteum is required.

One of the problems with the interpretation of MMP expression is that the detection of mRNA and protein, does not necessary mean active remodelling. These proteins require to be activated from their pro-enzyme precursors (Woessner, 1991; Birkedal-Hansen, 1995), and in their active form, they are inhibited by TIMPs. It is difficult to assess, in individual areas of the tissue, whether local MMP/TIMP ratios favour MMP action, or MMP inhibition. It is therefore difficult to accurately predict the action of MMPs in the presence of TIMPs. One of the ways to tackle this problem is to use a novel technique known as *in situ* zymography (Galis *et al.*, 1995). This technique allows the assessment of MMPs in their native environment. It allows for the detection of MMP activity in tissue sections containing the normal local levels of TIMPs. In addition, it does not result in the artificial activation of MMP pro-enzymes, that is evident when gelatine zymography is used (Salamonsen, 1996). This technique would give

valuable information about how the MMPs effect local tissue remodelling during luteolysis.

14.5.8 Endothelial Cells and Angiogenesis

In order to gain access to luteal steroidogenic cells, from the circulation, molecules have to cross the endothelial cell layer (Ghinea and Milgrom, 1995). The endothelial cells therefore have the potential to influence luteal function. The finding of IGFBP-3 in endothelial cells suggests they may be involved in the local control of IGF availability (Fraser *et al.*, 1997). In addition, luteal cells may influence endothelial cells. It is likely that the steroidogenic luteal cells are responsible for the production of angiogenic factors such as VEGF (Redmer *et al.*, 1996; Gordon *et al.*, 1996). It is also likely that luteal cells can produce vasoactive products such as NO and endothelin (Flores *et al.*, 1995; Kamada *et al.*, 1995; Magini *et al.*, 1996; Shiels *et al.*, 1996), that may have direct effects on endothelial function (Gross, 1995).

It is also possible that endothelial cell death can influence the survival and function of other luteal cells (Modlich *et al.*, 1996). As over 50% of cells in the mature corpus luteum are endothelial cells (Dharmarajan *et al.*, 1985), factors influencing their function have the ability to profoundly affect luteal cell function. To investigate luteal endothelial cells, several groups are now looking at these cells in primary culture (Spanel-Borowski, 1991; Bagavandoss and Wilks, 1991; Christenson and Stouffer, 1996b). Such an approach, combined with a steroidogenic cell co-culture, may reveal information about the endothelial-steroidogenic cell interaction and co-dependence.

Luteal angiogenesis is very marked (Reynolds *et al.*, 1992; Redmer and Reynolds, 1996), and groups involved in studying angiogenesis during neoplasia, are now increasingly aware of the corpus luteum as a model for angiogenesis. Recent studies have confirmed the high levels of angiogenesis and endothelial cell division during the early-luteal phase (Christenson and Stouffer, 1996; Rodger *et al.*, 1997). Indeed angiogenesis is fundamental to luteal function (Ferrara *et al.*, 1998). Disruption to angiogenesis has profound effects on luteal function in some models (Ferrara *et al.*, 1998). It is therefore useful to use the corpus luteum as a model to study luteal angiogenesis, angiogenesis in general, and the effects of inhibitors of angiogenesis (Folkman, 1985; Reynolds *et al.*, 1992). It is likely that luteal angiogenesis will be one of the most important areas of study in the future.

14.5.9 Novel Molecules

These approaches to studying the corpus luteum focus on recognised molecules. It is possible that novel, or unrecognised molecules are vital to the structure and function of the corpus luteum. At present, studies of the corpus luteum investigate molecules, and molecular processes, that have been shown to be important in other cellular systems, or relevant cell culture models. It is possible that the corpus luteum has some pathways or proteins which are either unique to the corpus luteum, or are much more important in the corpus luteum than in other systems. One method of detecting such proteins is to use comparative protein gels, at different, clearly defined, stages of the luteal phase. This is possible, by using the technique of two dimensional protein gel electrophoresis (McLaren *et al.*, 1994). This technique is commonly used to look at changes in protein production after damage to spermatogenesis (Turner *et al.*, 1996). Consistent changes in unspecified proteins throughout the luteal phase and after luteal 'rescue' can therefore by identified, and the proteins characterised.

In addition, total mRNA expression can be analysed, at different stages of the luteal phase, by differential display and subtractive hybridisation (Shima *et al.*, 1995; Wolf *et al.*, 1997; Jin *et al.*, 1997). This allows consistent differences in mRNA expression across the stages of the luteal phase to be detected and analysed. Using this technique, it is relatively straightforward to identify the nature of the consistently changing mRNA species. One of the attractive features of this approach is that, experiments using the well-characterised human luteal 'rescue' model, often reveal little change in protein, or mRNA, expression throughout the luteal phase (Rodger *et al.*, 1995; Duncan *et al.*, 1996a; Duncan *et al.*, 1996c; Rodger *et al.*, 1998). It is therefore likely that most mRNA species will not change consistently, and resources can be focused towards the ones which do. The disadvantages of these techniques, includes the fact that they are technically difficult, and expensive in terms of time, materials and equipment. Indeed, preliminary studies reveal some problems with reproducibility. In addition, funding bodies often look less favourably on techniques that are less clearly focused and involve more trial and error. Although these techniques are powerful, with great potential, it is likely that their application to the corpus luteum would be difficult.

However, although difficult, these techniques have the potential to highlight unexpected gene expression of fundamental import to luteal physiology, and even

general cellular biology. However, it remains possible that no helpful results would be detected. Subtractive hybridisation and differential display are novel methods for exploring the corpus luteum that may become increasingly important with continued technique refining. In addition, there is a clear role for the preparation of human luteal libraries. This would provide a necessary resource for the identification, and characterisation, of novel genes expressed in the corpus luteum. In addition, as the corpus luteum is a dynamic tissue, that has many globally important properties, it would would be an ideal source to form the basis of a powerful human library with general application.

14.6 Research Priorities

This thesis has described some elements which clearly change over the functional luteal phase. This is important, as many previous experiments have found little change in a variety of molecules studied (Rodger *et al.*, 1995; Duncan *et al.*, 1996a; Duncan *et al.*, 1996c; Rodger *et al.*, 1998). Therefore, these changes form a starting point to allow characterisation of the molecular changes controlling luteal function. An investigation into the nature of these changes, and a dissection of the causes, and their controls, should therefore be priorities for future related research. The two main areas of change were the influx of macrophages into the corpus luteum (Duncan *et al.,* 1998c), and the increased expression and activity of MMP-2 (Duncan *et al.,* 1998b). It is these areas which form the research priorities for the continuation of the work in the thesis.

14.6.1 Macrophage Influx

There is certainly an influx of macrophages into the human corpus luteum during luteolysis (Wang LJ *et al.*, 1992; Best *et al.*, 1996; Takaya *et al.*, 1997; Duncan *et al.*, 1998c), and this influx begins during the functional luteal phase. It is also likely that these macrophages have inhibitory effects on the function and structure of the corpus luteum (Norman and Brännström, 1994). However, it is not known what factors are involved in this influx of macrophages. What is now known, is that hCG during luteal 'rescue' prevents this influx (Duncan *et al.*, 1998c). As LH/hCG receptors are localised to the steroidogenic cells, it appears that steroidogenic cell products are involved in controlling macrophage influx. It is not

known whether hCG stimulates inhibitors of macrophage chemotaxis, or hCG inhibits the formation of chemotactic molecules.

Although many molecules, including IL-8 (Critchley *et al.*, 1994), are known to be chemoattractant, one of the major molecules involved in macrophage influx is MCP-1 (Leonard and Yoshimura, 1990). Studies on infraprimate corpora lutea have found the expression of MCP-1 (Townson *et al.*, 1996), and it seems to be elevated during macrophage influx (Bowen *et al.*, 1996; Naftalin *et al.*, 1997). At the moment MCP-1 is the best candidate molecule involved in macrophage influx. However, its expression in the primate remains to be fully investigated. A preliminary report has recently suggested that MCP-1 can, indeed, be detected in the human corpus luteum (Senturk *et al.*, 1997). However, a clear research priority is the systematic assessment of the expression and localisation of MCP-1 in the human corpus luteum, throughout the luteal phase and after luteal 'rescue' with exogenous hCG.

14.6.2 MMP-2 Expression

One of the factors which clearly changes during the transition between luteolysis and luteal 'rescue' is the expression of MMP-2 (Duncan *et al.*, 1998b). Expression of MMP-2 is, therefore, likely to have a role in the remodelling associated with luteolysis. However, the factors involved in its production are not clear. As MMP-2 distribution (Duncan *et al.*, 1998b) differs from that of the LH receptor (Duncan *et al.*, 1996a), it is likely that luteal cell products, rather than hCG, are involved in the control of its expression. It is also likely that the source of MMP-2 is the non-steroidogenic cells at the periphery of the corpus luteum (Duncan *et al.*, 1998b).

One approach to the investigation of MMP-2 expression, is the utilisation of a cell culture system. Establishment of luteal cells with the potential to express MMP would be important in this case. This has been achieved with luteinised granulosa cell culture (Puistola *et al.*, 1995; Aston *et al.*, 1996a). However, an attempt to culture non-steroidogenic stromal fibroblast-like cells from the corpus luteum may prove more useful. The effect of various luteal cell products including progesterone, IGFs, inhibin, activin and cytokines could be investigated. In addition, a search for steroid response elements in the MMP-2 gene, and the presence of steroid and protein receptors on the MMP-2-producing cells would give valuable additional information. The nature of the factors controlling MMP-2

expression should be one of the priorities for research on the primate corpus luteum.

14.7 Overall Conclusion

This thesis has used novel models of primate luteal function to investigate the corpus luteum throughout the luteal phase, after luteal 'rescue' with exogenous hCG, to simulate the hormonal changes of early pregnancy, and after induced luteolysis. Together, these models are unique, and have resulted in a bank of well characterised human, and non-human primate, luteal tissue, unsurpassed anywhere in the world. The use of these tissues has allowed insight into the changing nature of the steroidogenic and remodelling pathways throughout the primate luteal phase.

The findings reported in this thesis have been published in several peer reviewed journals (Duncan *et al.*, 1996a; Duncan *et al.*, 1996b; Duncan *et al.*, 1996c; Duncan *et al.*, 1998a; Duncan *et al.*, 1998b; Duncan *et al.*, 1998c). In addition, tissues collected using this regimen, have given valuable information in supplementary studies (Rodger *et al.*, 1995; Koh *et al.*, 1995; Illingworth *et al.*, 1996; Rodger *et al.*, 1997; Rodger *et al.*, 1998). In conclusion, the models used in these studies have provided valuable insights into the molecular control of the primate corpus luteum. They are powerful models which are unique. Research using these methods should continue. Speculative paradigms and new important areas of research have been developed and identified as a result of the studies reported in this thesis.

Chapter 15

References

Aatsinki JT, Pietilä EM, Lakkakorpi JT, Rajaniemi HJ (1992) Expression of the LH/CG receptor gene in rat ovarian tissue is regulated by an extensive alternative splicing of the primary transcript. *Mol. Cell. Endocrinol.* **84** 127-135.

Abayasekara DRE, Michael AE, Webley GE, Flint APF (1993) Mode of action of prostaglandin $F_{2\alpha}$ in human luteinized granulosa cells: role of protein kinase C. *Mol. Cell. Endocrinol.* **97** 81-91.

Abel MH (1985) Prostanoids and menstruation. In: Baird DT, Michel EA (eds), *Mechanisms of Menstrual Bleeding*, Serono Symposia. New York: Raven Press, pp139-156.

Adams EC, Hertig AT (1969) Studies of the human corpus luteum. I. Observations on the ultrastructure of development and regression of the luteal cells during the menstrual cycle. *Am. J. Cell Biol.* **41** 696-715.

Adashi EY (1990) The potential relevance of cytokines to ovarian physiology: the emerging role of resident ovarian cells of the white blood cell series. *Endocrine Rev.* **11** 454-464.

Adashi EY (1994) Endocrinology of the ovary. *Hum. Reprod.* **9** (Suppl. 2) 36-51.

Adashi EY (1995) Editorial: with a little help from my friends - the evolving story of intraovarian regulation. *Endocrinology* **136** 4161-4162.

Adashi EY, Resnick CE, Hernandez ER, Svoboda ME, Van Wyk JJ (1989) Potential relevance of insulin-like growth factor I to ovarian physiology: from basic science to clinical application. *Semin. Reprod. Endocrinol.* **7** 94-99.

Albertini DF, Anderson E (1974) The appearance and structure of intercellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions. *J. Cell Biol.* **63** 234-250.

Alila HW, Hansel W (1984) Induction of lymphopenia causes luteal dysfunction in cattle. *Biol. Reprod.* **31** 671-678.

Aloi JA, Dalkin AC, Schwartz NB, Yasin M, Mann B, Haisenleder DJ, Marshall JC (1995) Ovarian inhibin subunit gene expression: regulation by gonadotrophins and estradiol. *Endocrinology* **136** 1227-1232.

Alper MM, Sperling A, Penzias AS (1992) Laparoscopic salpingectomy using conventional laparoscopy equipment. *Int. J. Fertil.* **37** 26-28.

Amselgruber W, Sinowatz F, Schams D, Skottner A (1994) Immunohistochemical aspects of insulin-like growth factors I and II in the bovine corpus luteum. *J. Reprod. Fertil.* **101** 445-451.

Anderson L, Hillier SG, Eidne KA, Miro F (1996) GnRH-induced calcium mobilization and inositol phosphate production in immature and mature rat ovarian granulosa cells. *J. Endocrinol.* **149** 449-456.

Apa R, Fulghesu AM, Lanzone A, Guido M, Villa P, Pavone V, Caruso A, Mancuso S (1995) Effect of corticotrophin-releasing factor on the pituitary ovary axis in the human luteal phase. *Gynecol. Endocrinol.* **9** 271-276.

Arends MJ, Wyllie AH (1991) Apoptosis: mechanisms and roles in pathology. *Int. Rev. Exp. Path.* **32** 223-254.

Arici A, Oral E, Bukulmez O, Buradagunta S, Bahtiyar O, Jones EE (1997) Monocyte chemotactic protein-1 expression in human preovulatory follicles and ovarian cells. *J. Reprod. Immunol.* **32** 201-219.

Armstrong DT, Dorrington JH (1979) Estrogen biosynthesis in the ovaries and testes. In: Thomas JA, Singhal RL (eds), *Regulatory Mechanisms Affecting Gonadal Hormone Action*, Volume 2. Baltimore: University Park Press, pp217-258.

Asakura H, Zwain IH, Yen SSC (1997) Expression of genes encoding corticotropin-releasing factor (CRF), type I CRF receptor, and CRF-binding protein and localization of the gene products in the human ovary. *J. Clin. Endocrinol. Metab.* **82** 2720-2725.

Asch RH, Abou-Samara M, Braunstein GD, Pauerstein CJ (1982) Luteal function in hypophysectomized rhesus monkeys. *J. Clin. Endocrinol. Metab.* **55** 154-161.

Aston KE, Stamouli A, Thomas EJ, Vyas S, Iredale JP, Arthur MJP, Richardson MC (1996a) Effect of gonadotrophin on cell and matrix retention and expression of metalloproteinases and their inhibitor in cultured human granulosa cells modelling corpus luteum function. *Mol. Hum. Reprod.* **2** 26-30.

Aston KE, O'Sullivan MJB, Thomas EJ, Richardson MC (1996b) Effect of human chorionic gonadotrophin on the detachment of human granulosa cells from extracellular matrix layered onto glass or plastic. *Hum. Reprod.* **11** 336-340.

Auletta FJ, Flint APF (1988) Mechanisms controlling corpus luteum function in sheep, cows, nonhuman primates, and women especially in relation to the time of luteolysis. *Endocrine Rev.* **9** 88-105.

Auletta FJ, Kelm LB (1994) Mechanisms controlling corpus luteum function in the rhesus monkey (*Macaca mulatta*): inhibitory action of hCG on luteolysis induced by $PGF_{2\alpha}$. *J. Reprod. Fertil.* **102** 215-220.

Auletta FJ, Speroff L, Caldwell BV (1973) Prostaglandin $F_{2\alpha}$-induced steroidogenesis and luteolysis in the primate corpus luteum. *J. Clin. Endocrinol. Metab.* **36** 405-407.

Auletta FJ, Kamps DL, Pories S, Bisset J, Gibson M (1984a) An intra-corpus luteum site for the luteolytic action of $PGF_{2\alpha}$ in the rhesus monkey. *Prostaglandins* **27** 285-298.

Auletta FJ, Kamps DL, Wesley M, Gibson M (1984b) Luteolysis in the rhesus monkey: ovarian venous estrogen, progesterone, and prostaglandin $F_{2\alpha}$-metabolite. *Prostaglandins* **27** 299-310.

Auletta FJ, Paradis DK, Wesley M, Duby RT (1984c) Oxytocin is luteolytic in the rhesus monkey (*Macaca mulatta*). *J. Reprod. Fertil.* **72** 401-406.

Auletta FJ, Schofield MJ, Abae M (1990) The mechanisms controlling luteolysis in non-human primates and women. *Sem. Reprod. Endocrinol.* **8** 122-129.

Auletta FJ, Kelm LB, Schofield MJ (1995) Responsiveness of the corpus luteum of the rhesus monkey (*Macaca mulatta*) to gonadotropin *in vitro* during spontaneous and prostaglandin $F_{2\alpha}$-induced luteolysis. *J. Reprod. Fertil.* **103** 107-113.

Babra CS, Lynch SS, Foxcroft GR, Robinson G, Amoroso EC (1984) Purification and characterization of guinea-pig chorionic gonadotrophin. *J. Reprod. Fertil.* **71** 227-233.

Bacich DJ, Rohan RM, Norman RJ, Rodgers RJ (1994) Characterization and relative abundance of alternatively spliced luteinizing hormone receptor messenger ribonucleic acid in the ovine ovary. *Endocrinology* **135** 735-744.

Bagavandoss P, Wilks JW (1991) Isolation and characterization of microvascular endothelial cells from developing corpus luteum. *Biol. Reprod.* **44** 1132-1139.

Bagavandoss P, Midgley AR Jr, Wich M (1983) Developmental changes in the ovarian follicular basal lamina detected by immunofluorescence and electron microscopy. *J. Histochem. Cytochem.* **31** 633-640.

Bagavandoss P, Wiggins RC, Kunkel SL, Remick DG, Keyes PL (1990) Tumor necrosis factor production and accumulation of inflammatory cells in the corpus luteum of pseudopregnancy and pregnancy in rabbits. *Biol. Reprod.* **42** 367-376.

Baird DD, Weinberg CR, Wilcox AJ, McConnaughey DR, Musey PI, Collins DC (1991) Hormonal profiles of natural conception cycles ending in unrecognized pregnancy loss. *J. Clin. Endocrinol. Metab.* **72** 793-800.

Baird DT, Smith KB (1993) Inhibin and related peptides in the regulation of reproduction. *Oxf. Rev. Reprod. Biol.* **15** 191-232.

Baird DT, Bäckström T, McNeilly AS, Smith SK, Wathen CG (1984) Effect of enucleation of the corpus luteum at different stages of the luteal phase of the

human menstrual cycle on subsequent follicular development. *J. Reprod. Fertil.* **70** 615-624.

Balakier H, Stronell RD (1994) Color doppler assessment of folliculogenesis in *in vitro* fertilization patients. *Fertil. Steril.* **62** 1211-1216.

Balasch J, Vanrell JA (1987) Corpus luteum insufficiency and fertility - a matter of controversy. *Hum. Reprod.* **2** 557-567.

Balmaceda J, Asch RH, Fernandez EO, Valenzuela G, Eddy CA, Pauerstein CJ (1979) Prostaglandin production by rhesus monkey corpora lutea *in vitro*. *Fertil. Steril.* **31** 214-216.

Barnea ER, Oelsner G, Benveniste R, Romero R, DeCherney AH (1986) Progesterone, estradiol, and β-human chorionic gonadotropin secretion in patients with ectopic pregnancy. *J. Clin. Endocrinol. Metab.* **62** 529-531.

Bassett SG, Little-Ihrig LL, Mason JI, Zeleznik AJ (1991) Expression of messenger ribonucleic acids that encode for 3β-hydroxysteroid dehydrogenase and cholesterol side-chain cleavage enzyme throughout the luteal phase of the macaque menstrual cycle. *J. Clin. Endocrinol. Metab.* **72** 362-366.

Batista MC, Cartledge TP, Zellmer AW, Nieman LK, Loriaux DL, Merriam GR (1994) The antiprogestin RU486 delays the midcycle gonadotropin surge and ovulation in gonadotropin-releasing hormone-induced cycles. *Fertil. Steril.* **62** 28-34.

Battifora H, Mehta P (1993) Comparison of antibodies to progesterone receptor. *Appl. Immunohistochem.* **1** 83-84.

Behrman HR, Preston SL (1989) Luteolytic actions of peroxide in rat ovarian cells. *Endocrinology* **124** 2895-2900.

Behrman HR, Aten RF (1991) Evidence that hydrogen peroxide blocks hormone-sensitive cholesterol transport into mitochondria of rat luteal cells. *Endocrinology* **128** 2958-2966.

Behrman HR, Endo T, Aten RF, Musicki B (1993) Corpus luteum function and regression. *Reprod. Med. Rev.* **2** 153-180.

Bell SC (1983) Decidualization: regional differentiation and associated function. *Oxf. Rev. Reprod. Biol.* **5** 220-271.

Bennegard B, Hahlin M, Dennefors B (1987) Antigonadotropic effect of oxytocin on the isolated human corpus luteum. *Fertil. Steril.* **47** 431-435.

Best CL, Pudney J, Welch WR, Burger N, Hill JA (1996) Localization and characterization of white blood cell populations within the human ovary throughout the menstrual cycle and menopause. *Hum. Reprod.* **11** 790-797.

Bill CH II, Keyes PL (1983) 17β-estradiol maintains normal function of corpora lutea throughout pseudopregnancy in hypophysectomized rabbits. *Biol. Reprod.* **28** 608-617.

Birkedal-Hansen H (1995) Proteolytic remodeling of the extracellular matrix. *Curr. Opin. Cell Biol.* **7** 728-735.

Bjurulf E, Selstam G (1996) Rat luteinizing hormone receptor messenger ribonucleic acid expression and luteolysis: inhibition by prostaglandin $F_{2\alpha}$. *Biol. Reprod.* **54** 1350-1355.

Bjurulf E, Selstam G, Olofsson JL (1994) Increased LH receptor mRNA and extended corpus luteum function induced by prolactin and indomethacin treatment *in vitro* in hysterectomized pseudopregnant rats. *J. Reprod. Fertil.* **102** 139-145.

Bohnet HG, McNeilly AS (1979) Prolactin: assessment of its role in the human female. *Horm. Metab. Res.* **11** 533-546.

Boujrad N, Ogwuegbu SO, Garnier M, Lee C-H, Martin BM, Papadopoulos V (1995) Identification of a stimulator of steroid hormone synthesis isolated from testis. *Science* **268** 1609-1612.

Bourne TH, Hagstrom HG, Hahlin M, Josefsson B, Granberg S, Hellberg P, Hamberger L, Collins WP (1996) Ultrasound studies of vascular and morphological changes in the human corpus luteum during the menstrual cycle. *Fertil. Steril.* **65** 753-758.

Bowen JM, Keyes PL, Warren JS, Townson DH (1996) Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages. *Biol. Reprod.* **54** 1120-1127.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72** 248-254.

Bramley TA, Menzies GS (1988) Subcellular fractionation of the ovine corpus luteum: association of progesterone with ovine luteal membranes. *Mol. Cell. Endocrinol.* **59** 135-146.

Bramley TA, Menzies GS (1994) Particulate binding sites for steroid hormones in subcellular fractions of the ovine corpus luteum: properties and hormone specificity. *Mol. Cell. Endocrinol.* **103** 39-48.

Bramley TA, Stirling D, Swanston IA, Menzies GS, McNeilly AS, Baird DT (1987) Specific binding sites for gonadotrophin-releasing hormone, LH/chorionic gonadotrophin, low-density lipoprotein, prolactin and FSH in homogenates of human corpus luteum. II. Concentrations throughout the luteal phase of the menstrual cycle and early pregnancy. *J. Endocrinol.* **113** 317-327.

Brannian JD, Woodruff TK, Mather JP, Stouffer RL (1992) Activin A inhibits progesterone production by macaque luteal cells in culture. *J. Clin. Endocrinol. Metab.* **75** 756-761.

Brannian JD, Stouffer RL, Shiigi SM, Hoyer PB (1993) Isolation of ovine luteal cell subpopulations by flow cytometry. *Biol. Reprod.* **48** 495-502.

Brännström M, Norman RJ (1993) Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. *Hum. Reprod.* **8** 1762-1775.

Brännström M, Pascoe V, Norman RJ, McClure N (1994a) Localization of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. *Fertil. Steril.* **61** 488-495.

Brännström M, Giesecke L, Moore IC, Van Den Heuvel CJ, Robertson SA (1994b) Leukocyte subpopulations in the rat corpus luteum during pregnancy and pseudopregnancy. *Biol. Reprod.* **50** 1161-1167.

Brooks J, Crow WJ, McNeilly JR, McNeilly AS (1992) Relationship between gonadotropin subunit gene-expression, gonadotropin-releasing hormone receptor content and pituitary and plasma gonadotropin concentrations during the rebound release of FSH after treatment of ewes with bovine follicular-fluid during the luteal phase of the cycle. *J. Mol. Endocrinol.* **8** 109-118.

Browning JY, Keyes PL, Wolf RC (1980) Comparison of serum progesterone, 20α-hydroxy-progesterone and estradiol-17β in pregnant and pseudopregnant rabbits: evidence for postimplantation recognition of pregnancy. *Biol. Reprod.* **23** 1014-1019.

Bruce NW, Moor RM (1976) Capillary blood flow to ovarian follicles, stroma and corpora lutea of anesthetized sheep. *J. Reprod. Fertil.* **46** 299-304.

Bukovský A, Caudle MR, Keenan JA, Wimalasena J, Foster JS, Van Meter SE (1995) Quantitative evaluation of the cell cycle-related retinoblastoma protein and localization of Thy-1 differentiation protein and macrophages during follicular development and atresia and in human corpora lutea. *Biol. Reprod.* **52** 776-792.

Bukovský A, Caudle MR, Keenan JA, Wimalasena J, Upadhyaya NB, Van Meter SE (1996) Is irregular regression of corpora lutea in climacteric women caused by age-induced alterations in the tissue control system? *Am. J. Reprod. Immunol.* **36** 327-341.

Burger HG, Igarashi M (1988) Inhibin: Definition and nomenclature, including related substances. *J. Clin. Endocrinol. Metab.* **66** 885-886.

Butler WR, Hotchkiss J, Knobil E (1975) Functional luteolysis in the rhesus monkey: ovarian estrogen and progesterone during the luteal phase of the menstrual cycle. *Endocrinology* **96** 1509-1512.

Caldwell BV, Rotchell YE, Pang CY, Anderson GG, Kase N, Behrman HR (1980) Comparative study of high-dose chorionic gonadotropin on the human

and rat corpus luteum and effect of gonadotropin-releasing hormone on human luteal function. *Am. J. Obstet. Gynecol.* **136** 458-464.

Cameron JL, Stouffer RL (1982) Gonadotropin receptors of the primate corpus luteum. II. Changes in available luteinizing hormone- and chorionic gonadotropin-binding sites in macaque corpus luteal membranes during the nonfertile menstrual cycle. *Endocrinology* **110** 2068-2073.

Cameron VA, Nishimura E, Mathews L, Lewis KA, Sawchenko PE, Vale WW (1994) Hybridization histochemical localization of activin receptor subtypes in rat brain, pituitary, ovary, and testis. *Endocrinology* **134** 799-808.

Carr BR, MacDonald PC, Simpson ER (1982) The role of lipoproteins in the regulation of progesterone secretion by the corpus luteum. *Fertil. Steril.* **38** 303-311.

Challis JRG, Calder AA, Dilley S, Forster CS, Hillier K, Hunter DJS, Mackenzie IZ, Thorburn GD (1976) Production of prostaglandins E and F by corpora lutea, corpora albicantes and stroma from the human ovary. *J. Endocrinol.* **68** 401-408.

Chandrasekher YA, Brenner RM, Molskness TA, Yu Q, Stouffer RL (1991) Titrating luteinizing hormone surge requirements for ovulatory changes in primate follicles. II. Progesterone receptor expression in luteinizing granulosa cells. *J. Clin. Endocrinol. Metab.* **73** 584-589.

Chandrasekher YA, Melner MH, Nagalla SR, Stouffer RL (1994) Progesterone receptor, but not estradiol receptor, messenger ribonucleic acid is expressed in luteinizing granulosa cells and the corpus luteum in rhesus monkeys. *Endocrinology* **135** 307-314.

Chedrese PJ, The VL, Labrie F, Jourio AV, Murphy BD (1990) Evidence for the regulation of 3β-hydroxysteroid dehydrogenase messenger RNA by human chorionic gonadotropin in luteinized porcine granulosa cells. *Endocrinology* **126** 2228-2230.

Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162** 156-159.

Christenson LK, Stouffer RL (1996a) Proliferation of microvascular endothelial cells in the primate corpus luteum during the menstrual cycle and simulated early pregnancy. *Endocrinology* **137** 367-374.

Christenson LK, Stouffer RL (1996b) Isolation and culture of microvascular endothelial cells from the primate corpus luteum. *Biol. Reprod.* **55** 1397-1404.

Ciereszko RE, Guam Z, Stokes BT, Petroff BK, Ottobre AC, Ottobre JS (1995) Arachidonic acid inhibits hCG-stimulated progesterone production by corpora lutea of primates: potential mechanism of action. *Prostaglandins* **50** 103-119.

Clark BJ, Wells J, King SR, Stocco DM (1994) The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse leydig tumor cells. *J. Biol. Chem.* **269** 28314-28322.

Clark GM, McGuire WL, Hubay CA, Pearson OH, Marshall JS (1983) Progesterone Receptors as a prognostic factor in stage II breast cancer. *N. Engl. J. Med.* **309** 1343-1347.

Cole FE, Weed JC, Schneider GT, Holland JB, Geary WL, Rice BF (1973) The gonadotropin receptor of the human corpus luteum. *Am. J. Obstet. Gynecol.* **117** 87-95.

Conley AJ, Kaminski MA, Dubowsky SA, Jablonka-Shariff A, Redmer DA, Reynolds LP (1995) Immunohistochemical localization of 3β-hydroxysteroid dehydrogenase and P450 17α-hydroxylase during follicular and luteal development in pigs, sheep and cows. *Biol. Reprod.* **52** 1081-1094.

Cooke BA, Platts EA, Abayasekera R, Kurlak LO, Schulster D, Sullivan M (1989) Control of multiple transducing systems by LH which results in the modulation of adenylate cyclase, protein kinase C, lipoxygenases and cyclooxygenases. *J. Reprod. Fertil.* **37** (Suppl.) 139-145.

Corner GW (1943) *The Hormones in Human Reproduction.* Princeton: Princeton University Press.

Corner GW Jr (1956) The histological dating of the human corpus luteum of menstruation. *Am. J. Anat.* **98** 377-383.

Cowan BD (1997) Infertility evaluation. In: Cowan BD, Seifer DB (eds), *Clinical Reproductive Medicine*. Philadelphia: Lippincott-Raven, pp167-174.

Critchley HOD, Kelly RW, Kooy J (1994) Perivascular location of a chemokine interleukin-8 in human endometrium - a preliminary report. *Hum. Reprod.* **9** 1406-1409.

Crowley WF Jr, McArthur JW (1980) Stimulation of the normal menstrual cycle in Kallman's syndrome by pulsatile administration of luteinizing hormone-releasing hormone (LHRH). *J. Clin. Endocrinol. Metab.* **51** 173-175.

Croxatto HB, Salvatierra AM, Croxatto HD, Spitz IM (1989) Variable effects of RU486 on endometrial maintenance in the luteal phase extended by exogenous hCG. *Clin. Endocrinol.* **31** 15-23.

Csapo AI, Pulkkinen MO, Wiest WG (1973) Effects of luteectomy and progesterone replacement therapy in early pregnancy patients. *Am. J. Obstet. Gynecol.* **115** 759-765.

Cuevas P, Ying SY, Ling N, Ueno N, Esch F, Guillemin R (1987) Immunohistochemical detection of inhibin in the gonad. *Biochem. Biophys. Res. Comm.* **142** 23-30.

Curry TE, Mann JS, Huang MH, Keeble SC (1992) Gelatinase and proteoglycanase activity during the periovulatory period in the rat. *Biol. Reprod.* **46** 256-264.

Dabirashrafi H, Mohamad K, Behjatnia Y, Moghadami-Tabrizi N (1991) Adhesion formation after ovarian electrocauterization on patients with polycystic ovarian syndrome. *Fertil. Steril.* **55** 1200-1201.

Daly DC, Walters CA, Soto-Albors CE, Tohan N, Riddick DH (1984) A randomized study of dexamethasone in ovulation induction with clomiphene citrate. *Fertil. Steril.* **41** 844-848.

Dawood MY (1994) Corpus luteal insufficiency. *Curr. Opin. Obstet. Gynecol.* **6** 121-127.

Dawood MY, Khan-Dawood FS (1986) Human ovarian oxytocin: its source and relationship to steroid hormones. *Am. J. Obstet. Gynecol.* **154** 756-763.

Dawood MY, Khan-Dawood FS (1994) Human corpus luteum: human chorionic gonadotropin receptors during ectopic pregnancy. *Fertil. Steril.* **62** 711-715.

Deghenghi R, Boutignon F, Wuthrich P, Lenaerts V (1993) Antarelix (EP 24332) a novel water soluble LHRH antagonist. *Biomed. Pharmacol.* **47** 107-110.

Dennefors BL, Sjögren A, Hamberger L (1982) Progesterone and adenosine 3',5'-monophosphate formation by isolated human corpora lutea of different ages: influence of human chorionic gonadotropin and prostaglandins. *J. Clin. Endocrinol. Metab.* **55** 102-107.

Devoto L, Kohen P, Castro O, Vega M, Troncoso JL, Charreau E (1995) Multihormonal regulation of progesterone synthesis in cultured human midluteal cells. *J. Clin. Endocrinol. Metab.* **80** 1566-1570.

Dharmarajan AM, Bruce NW, Meyer GT (1985) Quantitative ultrastructural characteristics relating to transport between luteal cell cytoplasm and blood in the corpus luteum of the pregnant rat. *Am. J. Anat.* **172** 87-99.

Dharmarajan AM, Goodman SB, Tilly KI, Tilly JL (1994) Apoptosis during functional corpus luteum regression: evidence of a role for chorionic gonadotropin in promoting luteal cell survival. *Endocrine J.* **2** 295-303.

Djahanbakhch O, McNeilly AS, Hobson BM, Templeton AA (1981a) A rapid luteinizing hormone radioimmunoassay for the prediction of ovulation. *Br. J. Obstet. Gynaecol.* **88** 1016-1020.

Djahanbakhch O, Swanston IA, Corrie JET, McNeilly AS (1981b) Prediction of ovulation by progesterone. *Lancet* **ii** 1164-1165.

Doody KJ, Carr BR (1991) Diagnosis and treatment of luteal dysfunction. In: Hillier SG (ed), *Ovarian Endocrinology*. Boston: Blackwell Scientific Publications, pp260-318.

Doody KJ, Lorence MC, Mason JI, Simpson ER (1990) Expression of messenger ribonucleic acid species encoding steroidogenic enzymes in human

follicles and corpora lutea throughout the menstrual cycle. *J. Clin. Endocrinol. Metab.* **70** 1041-1045.

Douglas RH, Ginther OJ (1976) Concentrations of prostaglandin F in uterine venous plasma of anesthetized mares during the estrous cycle and in early pregnancy. *Prostaglandins* **11** 251-260.

Duffy DM, Stouffer RL (1995) Progesterone receptor messenger ribonucleic acid in the primate corpus luteum during the menstrual cycle: possible regulation by progesterone. *Endocrinology* **136** 1869-1876.

Duffy DM, Hess DL, Stouffer RL (1994) Acute administration of a 3β-hydroxysteroid dehydrogenase inhibitor to rhesus monkeys at the midluteal phase of thc menstrual cycle: evidence for possible autocrine regulation of the primate corpus luteum by progesterone. *J. Clin. Endocrinl. Metab.* **79** 1587-1594.

Duffy DM, Stouffer RL, Stewart DR (1995) Dissociation of relaxin and progesterone secretion from the primate corpus luteum by acute administration of a 3β-hydroxysteroid dehydrogenase inhibitor during the menstrual cycle. *Biol. Reprod.* **53** 447-453.

Duffy DM, Hutchison JS, Stewart DR, Stouffer RL (1996) Stimulation of primate luteal function by recombinant human chorionic gonadotropin and modulation of steroid, but not relaxin, production by an inhibitor of 3β-hydroxysteroid dehydrogenase during simulated early pregnancy. *J. Clin. Endocrinol. Metab.* **81** 2307-2313.

Duffy DM, Wells TR, Haluska GJ, Stouffer RL (1997) The ratio of progesterone receptor isoforms changes in the monkey corpus luteum during the luteal phase of the menstrual cycle. *Biol. Reprod.* **57** 693-699.

Duncan WC, Jenkins JM, Mathur RS Li TC (1994) Ovulation induction by human menopausal gonadotrophin.. *Contemp. Rev. Obstet. Gynaecol.* **6** 154-160.

Duncan WC, Sweeting VM, Cawood P, Illingworth PJ (1995) Measurement of creatine kinase activity and diagnosis of ectopic pregnancy. *Br. J. Obstet. Gynaecol.* **102** 233-237.

Duncan WC, McNeilly AS, Fraser HM, Illingworth PJ (1996a) Luteinizing hormone receptor in the human corpus luteum: lack of down-regulation during maternal recognition of pregnancy. *Hum. Reprod.* **11** 2291-2297.

Duncan WC, Illingworth PJ, Fraser HM (1996b) Expression of tissue inhibitor of metalloproteinases-1 in the primate ovary during induced luteal regression. *J. Endocrinol.* **151** 203-213.

Duncan WC, McNeilly AS, Illingworth PJ (1996c) Expression of tissue inhibitor of metalloproteinases-1 in the human corpus luteum after luteal rescue. *J. Endocrinol.* **148** 59-67.

Duncan WC, Illingworth PJ, Young FM, Fraser HM (1998a) Induced luteolysis in the primate: rapid loss of luteinizing hormone (LH) receptors. *Hum. Reprod.* In press.

Duncan WC, McNeilly AS, Illingworth PJ (1998b) The effect of luteal 'rescue' on the expression and localization of matrix metalloproteinases and their tissue inhibitors in the human corpus luteum. *J. Clin. Endocrinol. Metab.* In press.

Duncan WC, Rodger FE, Illingworth PJ (1998c) The human corpus luteum: reduction in macrophages during simulated maternal recognition of pregnancy. *Hum. Reprod.* In press.

Einspanier A, Ivell R, Rune G, Hodges JK (1994) Oxytocin gene expression and oxytocin immunoreactivity in the ovary of the common marmoset monkey (*Callithrix jacchus*). *Biol. Reprod.* **50** 1216-1222.

Ellinwood WE, Norman RL, Spies HG (1984) Changing frequency of pulsatile LH and progesterone secretion during the luteal phase of the menstrual cycle of rhesus monkeys. *Biol. Reprod.* **31** 714-722.

Emi N, Kanzaki H, Yoshida M, Takakura K, Kariya M, Okamoto H, Imai K, Mori T (1991) Lymphocytes stimulate progesterone production by cultured human granulosa luteal cells. *Am. J. Obstet. Gynecol.* **165** 1469-1474.

Emmi AM, Skurnick J, Goldsmith LT, Gagliardi CL, Schmidt CL, Kleinberg D, Weiss G (1991) Ovarian control of pituitary hormone secretion in early human pregnancy. *J. Clin. Endocrinol. Metab.* **72** 1359-1363.

Endo T, Aten RF, Wang F, Behrman HR (1993a) Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. *Endocrinology* **133** 690-698.

Endo T, Aten RF, Leykin L, Behrman HR (1993b) Hydrogen peroxide evokes antisteroidogenic and antigonadotropic actions in human granulosa-lutein cells. *J. Clin. Endocrinol. Metab.* **76** 337-342.

Eramaa M, Tuuri T, Hilden K, Ritvos O (1994) Regulation of inhibin α- and βA subunit messenger ribonucleic acid levels by chorionic gonadotropin and recombinant follicle stimulating hormone in cultured human granulosa-luteal cells. *J. Clin. Endocrinol. Metab.* **79** 1670-1677.

Eramaa M, Hilden K, Tuuri T, Ritvos O (1995) Regulation of inhibin/activin subunit messenger ribonucleic acids (mRNAs) by activin A and expression of activin receptor mRNAs in cultures of human granulosa-luteal cells. *Endocrinology* **136** 4382-4389.

Erden HF, Zwain IH, Asakura H, Yen SSC (1998) Corticotropin-releasing factor inhibits luteinizing hormone-stimulated $P450_{c17}$ gene expression and androgen production by isolated thecal cells of human ovarian follicles. *J. Clin. Endocrinol. Metab.* **83** 448-452.

Espey LL (1980) Ovulation as an inflammatory reaction - a hypothesis. *Biol. Reprod.* **22** 73-106.

Espey LL (1994) Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. *Biol. Reprod.* **50** 233-238.

Espey LL, Lipner H (1994) Ovulation. In: Knobil E, Neill JD (eds), *The Physiology of Reproduction*, Second Edition. New York: Raven Press Ltd., pp725-770.

Eyster KM, Ottobre JS, Stouffer RL (1985) Adenylate cyclase in the corpus luteum of the rhesus monkey. III. Changes in basal and gonadotropin-sensitive activities during the luteal phase of the menstrual cycle. *Endocrinology* **117** 1571-1577.

Ezra E, Salomon Y (1990) Mechanism of desensitization of adenylate cyclase by lutropin: GTP-dependent uncoupling of the receptor. *J. Biol. Chem.* **255** 653-658.

Fairchild Benyo D, Pate JL (1992) Tumor necrosis factor α alters bovine synthetic capacity and viability. *Endocrinology* **130** 854-860.

Fairchild Benyo D, Zeleznik AJ (1997) Cyclic adenosine monophosphate signalling in the primate corpus luteum: Maintenance of protein kinase A activity throughout the luteal phase of the menstrual cycle. *Endocrinology* **138** 3452-3458.

Ferrara N, Chen H, Davis-Smyth T, Gerber H-P, Nguyen T-N, Peers D, Chisholm V, Hillan KJ, Schwall RH (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nature Med.* **4** 336-340.

Findlay JK (1993) An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. *Biol. Reprod.* **48** 15-23.

Fisch B, Margara RA, Winston RML, Hillier SG (1990) Cellular basis of luteal steroidogenesis in the human ovary. *J. Endocrinol.* **122** 303-311.

Fishel SB, Edwards RG, Evans CJ (1984) Human chorionic gonadotropin secreted by preimplantation embryos cultured *in vitro*. *Science* **223** 816-818.

Fitz TA, Mayan MH, Sawyer HR, Niswender GD (1982) Characterization of two steroidogenic cell types in the ovine corpus luteum. *Biol. Reprod.* **27** 703-711.

Flores JA, Winters TA, Knight JW, Veldhuis JD (1995) Nature of endothelin binding in the porcine ovary. *Endocrinology* **136** 5014-5019.

Folkman J (1985) Tumour angiogenesis. *Adv. Cancer Res.* **43** 175-203.

Ford SP, Reynolds LP, Magness RR (1982) Blood flow to the uterine and ovarian vascular beds of gilts during the estrous cycle or early pregnancy. *Biol. Reprod.* **27** 878-885.

Fortune JE (1994) Ovarian follicular growth and development in mammals. *Biol. Reprod.* **50** 225-232.

Fowler RE, Fox NL, Edwards RG, Walters DE, Steptoe PC (1978) Steroidogenesis by cultured granulosa cells aspirated from human follicles using pregnenelone and androgens as precursors. *J. Endocrinol.* **77** 171-177.

Frank M, Bazer FW, Thatcher WW, Wilcox CJ (1977) A study of prostaglandin $F_{2\alpha}$ as the luteolysin in swine. III. Effects of estradiol valerate on prostaglandin F, progestin, estrone and estradiol concentration in the utero-ovarian veins of nonpregnant gilts. *Prostaglandins* **14** 1183-1196.

Fraser HM, Lunn SF (1993) Does inhibin have an endocrine function during the menstrual cycle? *Trends Endocrinol. Metab.* **4** 187-194.

Fraser HM, Abbott M, Laird NC, McNeilly AS, Nestor JJ, Vickery BM (1986) Effects of an LH-releasing hormone antagonist on the secretion of LH, FSH, prolactin and ovarian steroids at different stages of the luteal phase in the stumptailed macaque (*Macaca arctoides*). *J. Endocrinol.* **111** 83-89.

Fraser HM, Lunn SF, Cowen GM, Saunders PTK (1993) Localization of inhibin/activin subunit mRNAs during the luteal phase in the primate ovary. *J. Mol. Endocrinol.* **10** 245-257.

Fraser HM, Lunn SF, Whitelaw PF, Hillier SG (1995a) Induced luteal regression: differential effects on follicular and luteal inhibin/activin subunit mRNAs in the marmoset monkey. *J. Endocrinol.* **144** 201-208.

Fraser HM, Lunn SF, Cowen GM, Illingworth PJ (1995b) Induced luteal regression in the primate: evidence for apoptosis and changes in *c-myc* protein. *J. Endocrinol.* **147** 131-137.

Fraser HM, Lunn SF, Young FM, Eidne KA, Sellar R, Illingworth PJ, Harrison DJ (1995c) Apoptosis and necrosis after induced luteal regression in the primate. *Biol. Reprod.* **52** (Suppl. 1) 383.

Fraser HM, Lunn SF, Kim H, Erickson GF (1997) Localisation of insulin-like growth factor binding protein-3 (IGFBP-3) mRNA in the primate ovary. *Biol. Reprod.* **56** (Suppl. 1) 122.

Fukumatsu Y, Katabuchi H, Naito M, Takeya M, Takahashi K, Okamura H (1992) Effect of macrophages on proliferation of granulosa cells in the ovary in rats. *J. Reprod. Fertil.* **96** 241-249.

Fukuoka M, Yasuda K, Emi N, Fujiwara H, Iwai M, Takakura K, Kanzaki H, Mori T (1992) Cytokine modulation of progesterone and estradiol secretion in cultures of luteinized human granulosa cells. *J. Clin. Endocrinol. Metab.* **75** 254-258.

Galis ZS, Sukhova GK, Libby P (1995) Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. *FASEB J.* **9** 974-980.

Garren LD, Ney RL, Davis WW (1965) Studies on the role of protein synthesis in the regulation of corticosterone production by adrenocorticotrophic hormone *in vivo*. *Proc. Natl. Acad. Sci. USA* **53** 1443-1450.

Garzo VG, Liu J, Ulmann A, Baulieu E, Yen SSC (1988) Effects of an antiprogesterone (RU486) on the hypothalamic-hypophyseal-ovarian-endometrial axis during the luteal phase of the menstrual cycle. *J. Clin. Endocrinol. Metab.* **66** 508-517.

Gey GO, Seegar GE, Hellman LM (1938) The production of gonadotropic substance (Prolan) by placental cells in tissue culture. *Science* **88** 306-307.

Ghinea N, Milgrom E (1995) Transport of protein hormones through the vascular endothelium. *J. Endocrinol.* **145** 1-9.

Gibori G, Rothchild I, Pepe GJ, Morishige WK, Lam P (1974) Luteotropic action of decidual tissue in the rat. *Endocrinology* **85** 735-746.

Gillim SW, Christensen AK, McLennan CE (1969) Fine structure of the human menstrual corpus luteum at its stage of maximum secretory activity. *Am. J. Anat.* **126** 409-428.

Girsh E, Milvae RA, Wang W, Meidan R (1996) Effect of endothelin-1 on bovine luteal cell function - role in prostaglandin $F_{2\alpha}$-induced antisteroidogenic action. *Endocrinology* **137** 1306-1312.

Glock JL, Brumsted JR (1995) Color-flow pulsed doppler ultrasound in diagnosing luteal phase defect. *Fertil. Steril.* **64** 500-504.

Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S, He CS (1989) Human 72-kilodalton type IV collagenase forms a complex with a tissue

inhibitor of metalloproteinases designated TIMP-2. *Proc. Natl. Acad. Sci. USA* **86** 8207-8211

Goldstein JL, Brown MS (1977) The low-density lipoprotein pathway and its relation to atherosclerosis. *Ann. Rev. Biochem.* **46** 897-930.

Gordon JD, Mesiano S, Zaloudek CJ, Jaffe RB (1996) Vascular endothelial growth factor localization in human ovary and fallopian tubes: possible role in reproductive function and ovarian cyst formation. *J. Clin. Endocrinol. Metab.* **81** 353-359.

Gore BZ, Caldwell BV, Speroff L (1973) Estrogen-induced human luteolysis. *J. Clin. Endocrinol. Metab.* **36** 613-617.

Gougeon A (1979) Qualitative changes in medium and large antral follicles in the human ovary during the menstrual cycle. *Ann. Biol. Anim. Biochem. Biophys.* **19** 1461-1468.

Gougeon A (1996) Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocrine Rev.* **17** 121-155.

Graham JD, Clarke CL (1997) Physiological action of progesterone in target tissues. *Endocrine Rev.* **18** 502-519.

Graves PE, Pierce KL, Baily TJ, Rueda BR, Gil DW, Woodward DF, Yool AJ, Hoyer PB, Regan JW (1995) Cloning of a receptor for prostaglandin F2a from the ovine corpus luteum. *Endocrinology* **136** 3430-3436.

Greenberg LH, Stouffer RL, Brenner RM, Molskness TA, Hild-Petito SA, Yu Q (1990) Are human luteinizing granulosa cells a site of action for progesterone and relaxin? *Fertil. Steril.* **53** 446-453.

Grimes RW, Malton P, Ireland JJ (1987) A comparison of histological and non-histological indices of atresia and follicular function. *Biol. Reprod.* **37** 82-88.

Grody WW, Schrader WT, O'Malley BW (1982) Activation, transformation, and subunit structure of steroid hormone receptors. *Endocrine Rev.* **3** 141-163.

Groome NP, Illingworth PJ, O'Brien M, Priddle J, Weaver K, McNeilly AS (1995) Quantification of inhibin forms containing pro-αC in human serum by a new ultrasensitive ELISA. *J. Clin. Endocrinol. Metab.* **80** 2926-2932.

Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather J, McNeilly AS (1996) Measurement of dimeric inhibin B throughout the human menstrual cycle. *J. Clin. Endocrinol. Metab.* **81** 1401-1405.

Gross SS (1995) Nitric Oxide: pathophysiological mechanisms. *Ann. Rev. Physiol.* **57** 737-769.

Grusenmeyer DP, Pate JL (1992) Localization of prostaglandin $F_{2\alpha}$ inhibition of lipoprotein use by bovine luteal cells. *J. Reprod. Fertil.* **94** 311-318.

Guy MK, Juengel JL, Tandeski TR, Niswender GD (1995) Steady-state concentrations of messenger RNA encoding the receptor for luteinizing hormone during the estrous cycle and following prostaglandin $F_{2\alpha}$ treatment of ewes. *Endocrine* **3** 585-589.

Hagstrom HG, Hahlin M, Bennegardeden B, Bourne T, Hamberger L (1996) Regulation of corpus luteum function in early human pregnancy. *Fertil. Steril.* **65** 81-86.

Hahlin M, Dennefors B, Johanson C, Hamberger L (1988) Luteotropic effects of prostaglandin E_2 on the human corpus luteum of the menstrual cycle and early pregnancy. *J. Clin. Endocrinol. Metab.* **66** 909-914.

Hale AJ, Smith CA, Sutherland LC, Stoneman VEA, Longthorne VL, Culhane AC, Williams GT (1996) Apoptosis: molecular regulation of cell death. *Eur. J. Biochem.* **236** 1-26.

Hall PF (1985) Trophic stimulation of steroidogenesis: in search of the elusive trigger. *Rec. Prog. Horm. Res.* **41** 1-39.

Halme J, Hammond MG, Syrop CH, Talbert LM (1985) Peritoneal macrophages modulate human granulosa-luteal cell progesterone production. *J. Clin. Endocrinol. Metab.* **61** 912-916.

Hampton AL, Salamonsen LA (1994) Expression of messenger ribonucleic acid encoding matrix metalloproteinases and their tissue inhibitors is related to menstruation. *J. Endocrinol.* **141** R1-R3.

Hampton AL, Butt AR, Riley SC, Salamonsen LA (1995) Tissue inhibitors of metalloproteinases in endometrium of overiectomized steroid-treated ewes and during the estrous cycle and early pregnancy. *Biol. Reprod.* **53** 302-311.

Hanson FW, Powell JE, Stevens VC (1971) Effects of hCG and human pituitary LH on steroid secretion and functional life of the human corpus luteum. *J. Clin. Endocrinol.* **32** 211-215.

Hartung S, Rust W, Balvers M, Ivell R (1995) Molecular cloning and *in vivo* expression of the bovine steroidogenic acute regulatory protein. *Biochem. Biophys. Res. Comm.* **215** 646-653.

Hawkens DE, Belfiore CJ, Kile JP, Niswender GD (1993) Regulation of messenger ribonucleic acid encoding 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase in the ovine corpus luteum. *Biol. Reprod.* **48** 362-366.

Hay MF, Cran DG, Moor RM (1976) Structural changes occuring during atresia in sheep ovarian follicles. *Cell Tissue Res.* **169** 515-529.

Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K (1992) Growth promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. *FEBS Lett.* **298** 29-32.

Hearn JP (1986) The embryo-maternal dialogue during early pregnancy in primates. *J. Reprod. Fertil.* **76** 809-819.

Hearn JP, Webley GE (1987) Regulation of the corpus luteum of early pregnancy in the marmoset monkey - local interactions of luteotrophic and luteolytic hormones *in vivo* and their effects on the secretion of progesterone. *J. Endocrinol.* **114** 231-239.

Hearn JP, Abbot DM, Chambers PC, Hodges JK, Lunn SF (1978) Use of the common marmoset, *Callithrix jacchus*, in reproductive research. *Prim. Med.* **10** 40-49.

Hehnke KE, Christenson LK, Ford SP, Taylor M (1994) Macrophage infiltration into the porcine corpus luteum during prostaglandin $F_{2\alpha}$-induced luteolysis. *Biol. Reprod.* **50** 10-15.

Hernandez E, Hurwitz A, Vera A, Pellicer A, Adashi EY, LeRoith D, Roberts CT Jr (1992) Expression of the genes encoding the insulin-like growth factors and their receptors in the human ovary. *J. Clin. Endocrinol. Metab.* **74** 419-425.

Higuchi T, Kanzaki H, Nakayama H, Fujimoto M, Hatayama H, Kojima K, Iwai M, Mori T, Fujita J (1995) Induction of tissue inhibitor of metalloproteinase 3 gene expression during *in vitro* decidualization of human endometrial stromal cells. *Endocrinology* **136** 4973-4981.

Hild-Petito S, Fazleabas AT (1997) Expression of steroid receptors and steroidogenic enzymes in the baboon (*Papio anubis*) corpus luteum during the menstrual cycle and early pregnancy. *J. Clin. Endocrinol. Metab.* **82** 955-962.

Hild-Petito S, Stouffer RL, Brenner RM (1988) Immunohistochemical localisation of estradiol and progesterone receptors in the monkey ovary throughout the menstrual cycle. *Endocrinology* **123** 2896-2905.

Hillier SG (1985) Sex steroid metabolism and follicular development in the ovaries. *Oxf. Rev. Reprod. Biol.* **7** 168-222.

Hillier SG (1991) Paracrine control of follicular estrogen synthesis. *Sem. Reprod. Endocrinol.* **9** 332-340.

Hillier SG (1994) Current concept of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Hum. Reprod.* **9** 188-191.

Hillier SG, DeZwart FA (1981) Evidence that granulosa cell aromatase induction/activation by follicle stimulating hormone is an androgen receptor-regulated process *in vitro*. *Endocrinology* **109** 1303-1305.

Hillier SG, Zeleznik AJ, Knazek RA, Ross GT (1980) Hormonal regulation of preovulatory follicle maturation in the rat. *J. Reprod. Fertil.* **60** 219-229.

Hillier SG, Reichert LE Jr, van Hall EV (1981) Control of preovulatory follicular estrogen biosynthesis in the human ovary. *J. Clin. Endocrinol. Metab.* **52** 847-856.

Hillier SG, Yong EL, Illingworth PJ, Baird DT, Schwall RH, Mason AJ (1991a) Effect of recombinant inhibin on androgen synthesis in cultured human thecal cells. *Mol. Cell. Endocrinol.* **75** R1-R6.

Hillier SG, Wickings EJ, Illingworth PJ, Yong EL, Reichert LE Jr, Baird DT, McNeilly AS (1991b) Control of immunoactive inhibin production by human granulosa cells. *Clin. Endocrinol.* **35** 71-78.

Hinney B, Henze C, Kuhn W, Wuttke W (1996) The corpus luteum insufficiency - a multifactorial disease. *J. Clin. Endocrinol. Metab.* **81** 565-570.

Hirschfield AN (1984) Continuous [^{3}H] thymidine infusion: a method for the study of follicular dynamics. *Biol. Reprod.* **30** 485-491.

Hodgen GD (1983) Surrogate embryo transfer combined with estrogen-progesterone therapy in monkeys. Implantation, gestation and delivery without ovaries. *J.A.M.A.* **250** 2167-2172.

Hoeben E, Vanaelst I, Swinnen JV, Openakker G, Verhoeven G (1996) Gelatinase-A secretion and its control in peritubular and sertoli-cell cultures - effects of hormones, 2nd-messengers and inducers of cytokine production. *Mol. Cell. Endocrinol.* **118** 37-46

Hoffman YM, Peegel H, Sprock MJE, Zhang Q-Y, Menon KMJ (1991) Evidence that human chorionic gonadotropin/luteinizing hormone receptor-down regulation involves decreased levels of receptor message ribonucleic acid. *Endocrinology* **128** 338-393.

Honda T, Fujiwara H, Yamada S, Fujita K, Nakamura K, Nakayama T, Higuchi T, Ueda M, Maeda M, Mori T (1997) Integrin α5 is expressed on human luteinizing granulosa cells during corpus luteum formation, and its expression is enhanced by human chorionic gonadotrophin *in vitro*. *Mol. Hum. Reprod.* **3** 979-984.

Horie K, Takakura K, Fujiwara H, Lioa S, Mori T (1992) Immunohistochemical localization of androgen receptor in the human ovary throughout the menstrual cycle in relation to oestrogen and progesterone receptor expression. *Hum. Reprod.* **7** 184-190.

Horwitz KB, Wei LL, Sedlacek, SM, d'Arville CN (1985) Progestin action and progesterone receptor structure in human breast cancer: a review. *Recent Prog. Horm. Res.* **41** 249-316.

Hsueh AJW, Billig H, Tsafriri A (1994) Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocrine Rev.* **15** 707-724.

Huang J-C, Khan-Dawood FS, Dawood MY, Yeh J (1995) Baboon corpus luteum: epidermal growth factor receptor messenger ribonucleic acid expression during early, midluteal, and late luteal phases. *Fertil. Steril.* **63** 1318-1321.

Hulboy DL, Rudolph LA, Matrisian LM (1997) Matrix metalloproteinases as mediators of reproductive function. *Mol. Hum. Reprod.* **3** 27-45.

Hunter MG (1984) Prolactin stimulates steroidogenesis by human luteal tissue *in vitro*. *J. Endocrinol.* **103** 107-110.

Hurwitz A, Dushnik M, Solomon H, Ben-Chetrit A, Fini-Yeheskel Z, Milwidsky A, Mayer M, Adashi EY, Yagel S (1993) Cytokine-mediated regulation of rat ovarian function: interleukin-1 stimulates the accumulation of a 92-kilodalton gelatinase. *Endocrinology* **132** 2709-2714.

Hutchison JS, Zeleznik AJ (1984) The rhesus monkey corpus luteum is dependent on pituitary gonadotropin secretion throughout the luteal phase of the menstrual cycle. *Endocrinology* **115** 1780-1786.

Hutchison JS, Zeleznik AJ (1985) The corpus luteum of the primate menstrual cycle is capable of recovering from a transient withdrawal of pituitary gonadotropin support. *Endocrinology* **117** 1043-1049.

Hutchison JS, Nelson PB, Zeleznik AJ (1986) Effects of different gonadotrophin pulse frequencies on corpus luteum function during the menstrual cycle of rhesus monkeys. *Endocrinology* **119** 1964-1971.

Hutchison JS, Kubik CJ, Nelson PB, Zeleznik AJ (1987) Estrogen induces premature luteal regression in rhesus monkeys during spontaneous menstrual cycles but not in cycles driven by exogenous gonadotrophin-releasing hormone. *Endocrinology* **121** 466-474.

Iles RK, Chard T (1993) Molecular insights into the structure and function of human chorionic gonadotrophin. *J. Mol. Endocrinol.* **10** 217-234.

Illingworth PJ, Reddi K, Smith K, Baird DT (1990) Pharmacologic "rescue" of the corpus luteum results in increased inhibin production. *Clin. Endocrinol.* **33** 323-332.

Illingworth PJ, Reddi K, Smith K, Baird DT (1991) The source of inhibin secretion during the human menstrual cycle. *J. Clin. Endocrinol. Metab.* **73** 667-673.

Illingworth PJ, Groome NP, Duncan WC, Grant V, Tovanabutra S, Baird DT, McNeilly AS (1996) Measurement of circulating inhibin forms during the establishment of pregnancy. *J. Clin. Endocrinol. Metab.* **81** 1471-1475.

Imakawa K, Hansen TR, Malathy P-V, Anthony RV, Polites HG, Maroth KR, Roberts RM (1989) Molecular cloning and characterization of complementary deoxyribonucleic acids corresponding to bovine trophoblast protein-1: a comparison with ovine trophoblast protein-1 and bovine interferon-αII. *Mol. Endocrinol.* **3** 127-139.

Ivell R, Furuya K, Brackmann B, Dawood MY, Khan-Dawood FS (1990) Expression of the oxytocin and vasopressin genes in human and baboon gonadal tissues. *Endocrinology* **127** 2990-2996.

Iwai T, Nanbu Y, Iwai M, Taii S, Fujii S, Mori T (1990) Immunohistochemical localization of oestrogen receptors and progesterone receptors in the human ovary throughout the menstrual cycle. *Virchows Arch. A [Pathol. Anat. Histol.]* **417** 369-375.

Jacobson MD (1996) Reactive oxygen species and programmed cell death. *Trends Biological Sci.* **21** 83-86.

Jayatilak PG, Glasser LA, Warshaw ML, Herz Z, Gruber JR, Gibori G (1984) Relationship between luteinizing hormone and decidual luteotropin in the maintenance of luteal steroidogenesis. *Biol. Reprod.* **31** 556-564.

Ji I, Slaughter RG, Ellis JA, Ji TH, Murdoch WJ (1991) Analysis of ovine corpora lutea for tumor necrosis factor mRNA and bioactivity during prostaglandin-induced luteolysis. *Mol. Cell. Endocrinol.* **81** 77-80.

Jin H, Cheng X, Diatchenko L, Siebert PD, Huang CC (1997) Differential screening of a subtracted cDNA library: a method to search for genes preferentially expressed in multiple tissues. *Biotechniques* **23** 1084-1086.

Johnson MC, Devoto L, Retamales I, Kohen P, Troncoso JL, Aguilera G (1996) Localization of insulin-like growth factor (IGF-I) and IGF-I receptor expression in human corpora lutea - role on estradiol secretion. *Fertil. Steril.* **65** 489-494.

Johnson MR, Bolton VN, Riddle AF, Sharma V, Nicolaides K, Grudzinskas JG, Collins WP (1993) Interactions between the embryo and corpus luteum. *Hum. Reprod.* **8** 1496-1501.

Jones GS (1991) Luteal phase defect - a review of the pathophysiology. *Curr. Opin. Obstet. Gynecol.* **3** 641-648.

Jones JI, Clemmons DR (1995) Insulin-like growth factors and their binding proteins: Biological actions. *Endocrine Rev.* **16** 3-34.

Jones LS, Ottobre JS, Pate JL (1992) Progesterone regulation of luteinizing hormone receptors on cultured bovine luteal cells. *Mol. Cell. Endocrinol.* **85** 33-39.

Jordan AW (1981) Effects of prostaglandin $F_{2\alpha}$ treatment on LH and dibutyryl cyclic AMP-stimulated progesterone secretion by isolated rat luteal cells. *Biol. Reprod.* **25** 327-331.

Juengel JL, Garverick HA, Johnson AL, Youngquist RS, Smith MF (1993) Apoptosis during luteal regression in cattle. *Endocrinology* **132** 249-254.

Juengel JL, Smith GW, Smith MF, Youngquist RS, Garverick HA (1994) Pattern of protein production by bovine corpora lutea during luteolysis and

characterization of expression of two major secretory products of the regressing corpora lutea. *J. Reprod. Fertil.* **100** 515-520.

Juengel JL, Meberg BM, Turzillo AM, Nett TM, Niswender GD (1995) Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea. *Endocrinology* **136** 5423-5429.

Juengel JL, Wiltbank MC, Meberg BM, Niswender GD (1996) Regulation of steady state concentrations of messenger ribonucleic acid encoding prostaglandin $F_{2\alpha}$ receptor in ovine corpus luteum. *Biol. Reprod.* **54** 1096-1102.

Juengel JL, Nett TM, Anthony RV, Niswender GD (1997) Effects of luteotrophic and luteolytic hormones on expression of mRNA encoding insulin-like growth factor I and growth hormone receptor in the bovine corpus luteum. *J. Reprod. Fertil.* **110** 291-298.

Kamada S, Blackmore PF, Kubota T, Oehninger S, Asada Y, Gordon K, Hodgen GD, Aso T (1995) The role of endothelin-1 in regulating human granulosa cell proliferation and steroidogenesis *in vitro*. *J. Clin. Endocrinol. Metab.* **80** 3708-3714.

Karsch FJ, Sutton GP (1976) An intraovarian site for the luteotrophic action of estrogen in the rhesus monkey. *Endocrinology* **98** 553-561.

Karsch FJ, Krey LC, Weick RF, Dierschke DJ, Knobil E (1973) Functional luteolysis in the rhesus monkey: the role of estrogen. *Endocrinology* **92** 1148-1152.

Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J.* **9** 1603-1614.

Kato H, Sugino S, Takiguchi S, Kashida S, Nakamura Y (1997) Roles of reactive oxygen species in the regulation of luteal function. *Rev. Reprod.* **2** 81-83.

Kawaguchi K, Fujii S, Konishi I, Iwai T, Nabu Y, Nonogaki H, Ishikawa Y, Mori T (1991) Immunohistochemical analysis of oestrogen receptors, progesterone receptors and Ki67 in leiomyoma and myometrium during the menstrual cycle and pregnancy. *Virchows Arch. A [Pathol. Anat. Histol.]* **419** 309-315.

Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26** 239-257.

Khan MI, Rosberg S (1979) Acute suppression by $PGF_{2\alpha}$ on LH, epinephrine and fluoride stimulation of adenylate cyclase in rat luteal tissue. *J. Cyclic Nucleotide Res.* **5** 55-63.

Khan-Dawood FS (1997) The role of oxytocin in cell-to-cell communication in the corpus luteum. *Endocrinologist* **7** 160-166.

Khan-Dawood FS, Dawood MY (1983) Human ovaries contain immunoreactive oxytocin. *J. Clin. Endocrinol. Metab.* **57** 1129-1132.

Khan-Dawood FS, Marut EL, Dawood MY (1984) Oxytocin in the corpus luteum of the cynomolgus monkey *(Macaca fascicularis)*. *Endocrinology* **115** 570-574.

Khan-Dawood FS, Huang JC, Dawood MY (1988) Baboon corpus luteum oxytocin: an intragonadal peptide modulator of luteal function. *Am. J. Obstet. Gynecol.* **158** 882-891.

Khan-Dawood FS, Kanu EJ, Dawood MY (1993) Baboon corpus luteum: presence of oxytocin receptors. *Biol. Reprod.* **49** 262-266.

Khan-Dawood, Yang J, Anwer K, Dawood MY (1995) Bioactive oxytocin in human and baboon corpora lutea. *J. Endocrinol.* **147** 525-532.

Khan-Dawood FS, Dawood MY, Tabibzadeh S (1996a) Immunohistochemical analysis of the microanatomy of primate ovary. *Biol. Reprod.* **54** 734-742.

Khan-Dawood FS, Yang J, Dawood MY (1996b) Expression of gap junction protein connexin-43 in the human and baboon (*Papia anubis*) corpus luteum. *J. Clin. Endocrinol. Metab.* **81** 835-842.

Khan-Dawood FS, Yang J, Ozigi AA, Dawood MY (1996c) Immunocytochemical localization and expression of E-cadherin, β-catenin, and plakoglobin in the baboon (Papia anubis) corpus luteum. *Biol. Reprod.* **55** 485-491.

Kim-Björklund T, Landgren BM, Johannisson E (1991a) Morphometric studies of the endometrium, the fallopian tube and the corpus luteum during contraception with the 300 μg norethisterone (NET) minipill. *Contraception* **43** 459-474.

Kim-Björklund T, Landgren BM, Hamberger L, Johannisson E (1991b) Comparative morphometric study of the endometrium, the fallopian tube, and the corpus luteum during the postovulatory phase in normally menstruating women. *Fertil. Steril.* **56** 842-850.

King SR, Ronen-Fuhrmann T, Timberg R, Clark BJ, Orly J, Stocco DM (1995) Steroid production after *in vitro* transcription, translation, and mitochondrial processing of protein products of complementary deoxyribonucleic acid for steroidogenic acute regulatory protein. *Endocrinology* **136** 5165-5176.

Kiriakidou M, McAllister JM, Sugawara T, Strauss JF III (1996) Expression of steroidogenic acute regulatory protein (StAR) in the human ovary. *J. Clin. Endocrinol. Metab.* **81** 4122-4128.

Kirsch TM, Friedman AC, Vogel RL, Flickinger GL (1981) Macrophages in corpora lutea of mice: characterization and effects on steroid secretion. *Biol. Reprod.* **25** 629-638.

Kirsch TM, Vogel RL, Flickinger GL (1983) Macrophages: a source of luteotrophic cybernins. *Endocrinology* **115** 1910-1912.

Klagsbrun MA, D'Amore PA (1991) Regulators of angiogenesis. *Ann. Rev. Physiol.* **53** 217-239.

Knobil E (1973) On the regulation of the primate corpus luteum. *Biol. Reprod.* **8** 246-258.

Koh EAT, Illingworth PJ, Duncan WC, Critchley HOD (1995) Immunolocalization of bcl-2 protein in human endometrium in the menstrual cycle and simulated early pregnancy. *Hum. Reprod.* **10** 1557-1562.

Kupesic S, Kurjak A (1997) The assessment of normal and abnormal luteal function by transvaginal color doppler sonography. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **72** 83-87.

Lake S, Gullberg H, Wahlqvist J, Sjogren AM, Kinhult A, Lind P, Hellstromlindahl E, Stjernschantz J (1994) Cloning of the rat and human prostaglandin $F_{2\alpha}$ receptors and the expression of the rat prostaglandin $F_{2\alpha}$ receptor. *FEBS Lett.* **355** 317-325.

LaPolt P, Oikawa M, Jia X-C, Dargan C, Hsueh AJW (1990) Gonadotropin-induced up- and down-regulation of rat ovarian LH receptor message levels during follicular growth, ovulation and luteinization. *Endocrinology* **126** 3277-3279.

Leco KJ, Khokha R, Pavloff N, Hawkes SP, Edwards DR (1994) Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J. Biol. Chem.* **269** 9352-9360.

Ledger WL, Sweeting VM, Chaterjee S (1994) Rapid diagnosis of early ectopic pregnancy in an emergency gynaecology service - are measurements of progesterone and intact and free β human chorionic gonadotrophin helpful? *Hum. Reprod.* **9** 157-160.

Lee C, Keyes PL, Jacobson HI (1971) Estrogen receptor in the rabbit corpus luteum. *Science* **173** 1031-1033.

Lei ZM, Chegini N, Rao Ch V (1991) Quantitative cell composition of human and bovine corpora lutea from various reproductive states. *Biol. Reprod.* **44** 1148-1156.

Leith GS, Ginther OJ (1984) Characterization of intrauterine mobility of the early equine conceptus. *Theriogeneology* **22** 401-408.

LeMaire WJ, Marsh JM (1975) Interrelationships between prostaglandins, cyclic AMP and steroids in ovulation. *J. Reprod. Fertil.* **22** (Suppl.) 53-74.

Lenton EA, Woodward AJ (1988) The endocrinology of conception cycles and implantation in women. *J. Reprod. Fertil.* **36** (Suppl.) 1-15.

Leonard EJ, Yoshimura T (1990) Human monocyte chemoattractant protein-1 (MCP-1). *Immunol. Today* **11** 97-101.

Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KS (1988) Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J. Clin. Endocrinol. Metab.* **67** 334-340.

Li R, Phillips DM, Mather JP (1995) Activin promotes ovarian follicle development *in vitro*. *Endocrinology* **136** 849-856.

Li TC, Rogers AW, Dockery P, Lenton EA, Cooke ID (1988) A new method of histologic dating of human endometrium in the luteal phase. *Fertil. Steril.* **50** 52-60.

Li TC, Warren MA, Dockery P, Cooke ID (1991a) Human endometrial morphology around the time of implantation in natural and artificial cycles. *J. Reprod. Fertil.* **92** 543-554.

Li TC, Tristram A, Hill AS, Cooke ID (1991b) A review of 254 ectopic pregnancies in a teaching hospital in the Trent Region, 1977-1990. *Hum. Reprod.* **6** 1002-1007.

Lin D, Sugawara T, Strauss JF III, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL (1995) Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267** 1828-1831.

Liu K, Brandstrom A, Lui YX, Ny T, Selstam G (1996) Coordinated expression of tissue type plasminogen activator type I during corpus luteum formation and luteolysis in the adult pseudopregnant rat. *Endocrinology* **137** 2126-2132.

López Bernal A, Bellinger J, Marshall JM, Phaneuf S, Europe-Finner GN, Asbóth G, Barlow DH (1995) G protein expression and second messenger formation in human granulosa cells. *J. Reprod. Fertil.* **104** 77-83.

López Diaz MC, Bosu WTK (1997) Effects of ACTH on luteinizing hormone receptors in ovine follicular wall and corpus luteum. *Reprod. Nut. Dev.* **37** 599-612.

Lower AM, Yovich JL, Hancock C, Grudzinskas JG (1993) Is luteal function maintained by factors other than chorionic gonadotrophin in early pregnancy? *Hum. Reprod.* **8** 645-648.

Lu DL, Peegel H, Mosier SM, Menon KMJ (1993) Loss of lutropin/human choriogonadotropin receptor messenger ribonucleic acid during ligand-induced downregulation occurs post transcriptionally. *Endocrinology* **132** 235-240.

Luck MR, Zhao Y (1995) Structural remodelling of reproductive tissues. *J. Endocrinol.* **146** 191-195.

Luconi M, Bonaccorsi L, Maggi N, Pecchioli P, Krausz C, Forti G, Baldi E (1998) Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. *J. Clin. Endocrinol. Metab.* **83** 877-885.

Lunn SF (1998) The relative merits of the marmoset as a model in reproductive medicine. In: Pryce C, Scott L, Schenell C (eds), *Marmosets and Tamarins in Biological and Biomedical Research.* Salisbury: DSSD Imagery, pp192-207.

Lutjen P, Trounson AO, Leeton J, Findlay J, Wood C, Renou P (1984) The establishment and maintenance of pregnancies using *in vitro* fertilization and embryo donation in a patient with primary ovarian failure. *Nature* **307** 174-175.

Lytton FDC, Poyser NL (1982) Prostaglandin production by the rabbit uterus and placenta *in vitro*. *J. Reprod. Fertil.* **66** 591-599.

MacLennan AH, Grant P, Borthwick AC (1991) Relaxin and relaxin C-peptide levels in human reproductive tissues. *Reprod. Fertil. Dev.* **3** 577-583.

Magini A, Granchi S, Orlando C, Vannelli GB, Pellegrini S, Milani S, Grappone C, De Franco R, Susini T, Forti G, Maggi M (1996) Expression of endothelin-1 gene and protein in human granulosa cells. *J. Clin. Endocrinol. Metab.* **81** 1428-1433.

Malven PV (1969) Hypophysial regulation of luteolysis in the rat. In: McKerns KW (ed) *The Gonads*. New York: Meredith Corporation, pp367-382.

Mamluk R, Chen DB, Greber Y, Davis JS, Meidan R (1998) Characterization of messenger ribonucleic acid expression for prostaglandin $F_{2\alpha}$ and luteinizing hormone receptors in various bovine luteal cell types. *Biol. Reprod.* **58** 849-856.

Manaugh LC, Novy MJ (1976) Effects of indomethacin on corpus luteum function and pregnancy in rhesus monkeys. *Fertil. Steril.* **27** 588-598.

Mann JS, Kindy MS, Edwards DR, Curry TE Jr (1991) Hormonal regulation of matrix metalloproteinase inhibitors in rat granulosa cells and ovaries. *Endocrinology* **128** 1825-1832.

Margolin Y, Aten RF, Behrman HR (1990) Antigonadotropic and antisteroidogenic actions of peroxide in rat granulosa cells. *Endocrinology* **127** 245-250.

Marsh JM (1976) The role of cAMP in gonadal steroidogenesis. *Biol. Reprod.* **14** 30-53.

Matrisian LM (1990) Metalloproteinases and their inhibitors in matrix remodelling. *Trends Genet.* **6** 121-125.

Matzuk MM, Finegold MJ, Su JGJ, Hsueh AJW, Bradley A (1992) α-Inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature* **360** 313-319.

McClure N, Macpherson AM, Healy DL, Wreford N, Rogers PAW (1994) An immunohistochemical study of the vascularization of the human Graafian follicle. *Hum. Reprod.* **9** 1401-1405.

McLachlan RI, Robertson DM, Healy DL, Burger HG, deKretser DM (1987) Circulating immunoreactive inhibin levels during the normal human menstrual cycle. *J. Clin. Endocrinol. Metab.* **65** 954-961.

McLaren TT, Foster PMD, Sharpe RM (1994) Identification of stage-specific changes in protein secretion by isolated seminiferous tubules from rats

following exposure to short-term local testicular heating. *J. Reprod. Fertil.* **102** 293-300.

McLean MP, Nelson SE, Billheimer JT, Gibori S (1992) Differential capacity for cholesterol transport and processing in large and small rat luteal cells. *Endocrinology* **131** 2203-2212.

McLean MP, Billheimer JT, Warden KJ, Irby RB (1995) Prostaglandin $F_{2\alpha}$ mediates ovarian sterol carrier protein-2 expression during luteolysis. *Endocrinology* **136** 4963-4972.

McNatty KP, Makris A, DeGrazia C, Osathanondh R, Ryan KJ (1979) The production of progesterone, androgens, and estrogens by granulosa cells, thecal tissue, and stromal tissue from human ovaries *in vitro*. *J. Clin. Endocrinol. Metab.* **49** 687-699.

McNeilly AS, Kerin J, Swanston IA, Bramley TA, Baird DT (1980) Changes in the binding of human chorionic gonadotrophin/luteinizing hormone, follicle-stimulating hormone and prolactin to human corpora lutea during the menstrual cycle and pregnancy. *J. Endocrinol.* **87** 315-325.

Menzies GS, Bramley TA (1994) Specific binding sites for progesterone in subcellular fractions of the porcine corpus luteum. *J. Endocrinol.* **142** 101-110.

Michael AE, Abayasekara DRE, Webley GE (1994) Cellular mechanisms of luteolysis. *Mol. Cell. Endocrinol.* **99** R1-R9.

Millar MR, Sharpe RM, Maguire SM, Gaughan J, Saunders PTK (1995) Co-localization of messenger RNA and protein using *in situ* hybridization and immunohistochemistry in testicular tissue. *Microscopy Res. Technique* **32** 498-503.

Miller WL (1988) Molecular biology of steroid hormone synthesis. *Endocrine Rev.* **9** 295-318.

Minegishi T, Nakamura K, Takakura Y, Miyamoto K, Hasegawa Y, Ibuki Y, Igarashi M (1990) Cloning and sequencing of human LH/hCG receptor cDNA. *Biochem. Biophys. Res. Comm.* **172** 1049-1054.

Minegishi T, Tano M, Abe Y, Nakamura K, Ibuki Y, Miyamoto K (1997) Expression of luteinizing hormone/human chorionic gonadotrophin (LH/hCG) receptor mRNA in the human ovary. *Mol. Hum. Reprod.* **3** 101-107.

Miro F, Hillier SC (1992) Relative effects of activin and inhibin on steroid hormone synthesis in primate granulosa cells. *J. Clin. Endocrinol. Metab.* **75** 1556-1561.

Mitchell DE, Lei ZM, Rao ChV (1991) The enzymes in cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism in the human corpus luteum: dependence on luteal phase, cellular and subcellular distribution. *Prost. Leukotrienes Ess. Fatty Acids* **43** 1-12.

Modlich U, Kaup F-J, Augustin HG (1996) Cyclic angiogenesis and blood vessel regression in the ovary: blood vessel regression during luteolysis involves endothelial cell detachment and vessel occlusion. *Lab. Invest.* **74** 771-780.

Molenaar P, O'Reilly G, Sharkey A, Kuc RE, Harding DP, Plumpton C, Gresham GA, Davenport AP (1993) Characterization and localization of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium. *Circulation Res.* **72** 526-538.

Morell AG, Gregoriadis G, Scheinberg IH (1971) The role of sialic acid in determining the survival of gonadotropins in the circulation. *J. Biol. Chem.* **246** 1461-1467.

Morgan A, Keeble SC, London SN, Mase KN, Curry TE Jr (1994) Antiprogesterone (RU486) effects on metalloproteinase inhibitor activity in human and rat granulosa cells. *Fertil. Steril.* **61** 949-955.

Murdoch WJ (1987) Treatment of sheep with prostaglandin $F_{2\alpha}$ enhances production of a luteal chemoattractant for eosinophils. *Am. J. Reprod. Immunol. Microbiol.* **15** 52-56.

Murphy G, Ward R, Hembry RM, Raynolds JJ, Kühn K, Tryggvason K (1989) Characterization of gelatinase from pig polymorphonuclear leucocytes. *Biochem. J.* **258** 463-472.

Musicki B, Aten RF, Behrman HR (1993) Inhibition of protein synthesis and hormone-sensitive steroidogenesis in response to hydrogen peroxide in rat luteal cells. *Endocrinology* **134** 588-595.

Muttukrishna S, Fowler PA, George L, Groome NP, Knight PJ (1996) Changes in peripheral serum levels of total activin-A during the human menstrual cycle and pregnancy. *J. Clin. Endocrinol. Metab.* **81** 3328-3334.

Naether OGJ, Fischer R (1993) Adhesion formation after laparoscopic electrocoagulation of the surface in polycystic ovary patients. *Fertil. Steril.* **60** 95-98.

Naftalin DM, Bove SE, Keyes PL, Townson DH (1997) Estrogen withdrawal induces macrophage invasion in the rabbit corpus luteum. *Biol. Reprod.* **56** 1175-1180.

Nappi C, Gargiulo AR, Di Carlo C (1994) The human luteal paracrine system: current concepts. *J. Endocrinol. Invest.* **17** 825-836.

Naylor MS, Stamp GW, Davies BD, Balkwill FR (1994) Expression and activity of MMPs and their regulators in ovarian cancer. *Int. J. Cancer* **58** 50-56.

Neill JD, Johansson ED, Knobil E (1969a) Failure of hysterectomy to influence the normal pattern of cyclic progesterone secretion in the rhesus monkey. *Endocrinology* **84** 464-467.

Neill JD, Johansson EDB, Knobil E (1969b) Patterns of circulating progesterone concentrations during the fertile menstrual cycle and the remainder of gestation in the rhesus monkey. *Endocrinology* **84** 45-48.

Nelson SE, McLean MP, Jayatilak PG, Gibori G (1992) Isolation, characterization, and culture of cell subpopulations forming the pregnant rat corpus luteum. *Endocrinology* **130** 954-966.

Nett TM, McClellan MC, Niswender GD (1976) Effects of prostaglandins on the ovine corpus luteum: blood flow, secretion of progesterone and morphology. *Biol. Reprod.* **15** 66-78.

Nicola NA (1989) Hemopoietic cell growth factors and their receptors. *Ann. Rev. Biochem.* **58** 45-77.

Nikkari ST, Hoyhtya M, Isola J, Nikkari T (1996) Macrophages contain 92-kD gelatinase (MMP-9) at the site of degenerated internal elastic lamina in temporal arteritis. *Am. J. Pathol.* **149** 1427-1433.

Nishihara T, Ohsaki Y, Ueda N, Koseki T, Eto Y (1995) Induction of apoptosis in B-lineage cells by activin-A derived from macrophages. *J. Interferon Cytokine Res.* **15** 509-516.

Nishimori K, Dunkel L, Hsueh AJW, Yamoto M, Nakano R (1995) Expression of luteinizing hormone and chorionic gonadotropin receptor messenger ribonucleic acid in human corpora lutea during menstrual cycle and pregnancy. *J. Clin. Endocrinol. Metab.* **80** 1444-1448.

Nishizuka Y, Sakakura T (1969) Thymus and Reproduction: sex-linked dysgenesis of the gonad after neonatal thymectomy of mice. *Science* **166** 753-755.

Niswender GD, Nett TM (1994) The corpus luteum and its control in infraprimate species. In: Knobil E, Neill JD (eds), *The Physiology of Reproduction*, Second edition. New York: Raven Press, pp781-816.

Niswender GD, Reimers TJ, Diekman MA, Nett TM (1976) Blood flow: a mediator of ovarian function. *Biol. Reprod.* **14** 64-81.

Niswender GD, Schwall RH, Fitz TA, Farin CE, Sawyer HR (1985) Regulation of luteal function in domestic ruminants: new concepts. *Rec. Prog. Hormone Res.* **41** 101-151.

Niswender GD, Juengel JL, McGuire WJ, Belfiore CJ, Wiltbank MC (1994) Luteal function: the estrous cycle and early pregnancy. *Biol. Reprod.* **50** 239-247.

Norman RJ, Brännström M (1994) White cells and the ovary - incidental invaders or essential effectors. *J. Endocrinol.* **140** 333-336.

Nothnick WB, Curry TE Jr (1996) Divergent effects of interleukin-1β on steroidogenesis and matrix metalloproteinase inhibitor expression and activity in cultured rat granulosa cells. *Endocrinology* **137** 3784-3790.

Nothnick WB, Edwards DR, Leco KJ, Curry TE Jr (1995) Expression and activity of ovarian tissue inhibitors of metalloproteinases during pseudopregnancy in the rat. *Biol. Reprod.* **53** 684-691.

Nothnick WB, Keeble SC, Curry TE Jr (1996) Collagenase, gelatinase, and proteoglycanase messenger ribonucleic acid expression and activity during luteal development, maintenance, and regression in the pseudopregnant rat ovary. *Biol. Reprod.* **54** 616-624.

Nothnick WB, Soloway P, Curry TE Jr (1997) Assessment of the role of tissue inhibitor of metalloproteinase-1 (TIMP-1) during the periovulatory period in female mice lacking a functional TIMP-1 gene. *Biol. Reprod.* **56** 1181-1188.

Obasiolu CCW, Khan-Dawood FS, Dawood MY (1992) Insulin-like growth factor-I receptors in human corpora lutea. *Fertil. Steril.* **57** 1235-1240.

Okuma A, Kuraoka A, Iida H, Inai T, Wasano K, Shibata Y (1996) Co-localization of connexin 43 and connexin 45 but absence of Connexin 40 in granulosa cell gap junctions in the rat ovary. *J. Reprod. Fertil.* **107** 255-264.

Olive DL (1991) The prevalence and epidemiology of luteal phase deficiency in normal and infertile women. *Clin. Obstet. Gynecol.* **34** 157-166.

Olofsson J, Leung PCK (1994) Auto/paracrine role of prostaglandins in corpus luteum function. *Mol. Cell. Endocrinol.* **100** 87-91.

Olofsson J, Norjavaara E, Selstam G (1990) *In vivo* levels of prostaglandin $F_{2\alpha}$, E_2 and prostacyclin in the corpus luteum of pregnant and pseudopregnant rats. *Biol. Reprod.* **42** 792-768.

Olofsson JI, Leung CHB, Bjurulf E, Ohno T, Selstam G, Peng C, Leung PCK (1996) Characterization and regulation of a messenger RNA encoding the prostaglandin $F_{2\alpha}$ receptor in the rat ovary. *Mol. Cell. Endocrinol.* **123** 45-52.

Oonk RB, Krasnow JS, Beattie WG, Richards JS (1989) Cyclic AMP-dependent and independent regulation of cholesterol side chain cleavage

cytochrome P-450 in rat ovarian granulosa cells and corpora lutea. *J. Biol. Chem.* **264** 21934-21942.

Oppenheim JJ, Zachariae COC, Mukaida N, Matsushima K (1991) Properties of the novel proinflammatory supergene "intracrine" cytokine family. *Ann. Rev. Immunol.* **9** 617-648.

O'Riordan JHL, Malan PG, Gould RP (1982) *Essentials of Endocrinology.* Oxford: Blackwell Scientific Publications.

Ottobre JS, Stouffer RL (1986) Receptors for chorionic gonadotropin in the corpus luteum of the rhesus monkey during simulated early pregnancy: lack of down-regulation. *Endocrinology* **119** 1594-1602.

Ottobre JS, Ottobre AC, Stouffer RL (1984) Changes in available gonadotropin receptors in the corpus luteum of the rhesus monkey during simulated early pregnancy. *Endocrinology* **115** 198-204.

Paavola LG (1979) The corpus luteum of the guinea pig. IV. Fine structure of macrophages during pregnancy and postpartum luteolysis and the phagocytosis of luteal cells. *Am. J. Anat.* **50** 337-364.

Pache TD, Wladimiroff JW, de Jong FH, Hop WC, Fauser BCJM (1990) Growth patterns of non-dominant ovarian follicles during the normal menstrual cycle. *Fertil. Steril.* **54** 638-642.

Pakarinen P, Vihko KK, Voutilainen R, Huhtaniemi I (1990) Differential response of luteinizing hormone receptor and steroidogenic enzyme gene expression to human chorionic gonadotropin stimulation in the neonatal and adult rat testis. *Endocrinology* **127** 2469-2474.

Parke-Sarge O-K, Parmer TG, Gu Y, Gibori G (1995) Does the rat corpus luteum express the progesterone receptor gene? *Endocrinology* **136** 1537-1545.

Patwardhan VV, Lanthier A (1980) Concentration of prostaglandins, PGE, PGF, estrone, estradiol, and progesterone in human corpora lutea. *Prostaglandins* **20** 963-969.

Pederson ES (1951) Histogenesis of lutein tissue of the albino rat. *Am. J. Anat.* **88** 397-427.

Peegel H, Randolph J Jr, Midgley AR, Menon KMJ (1994) *In situ* hybridization of luteinizing hormone/human chorionic gonadotropin receptor messenger ribonucleic acid during hormone-induced down-regulation and the subsequent recovery in rat corpus luteum. *Endocrinology* **135** 1044-1051.

Penney GC, Souter V, Glasier A, Templeton AA (1997) Laparoscopic sterilisation: Opinion and practice among gynaecologists in Scotland. *Br. J. Obstet. Gynaecol.* **104** 71-77.

Perkes CM, Wathes DC (1996) Expression of mRNAs for insulin-like growth factor binding proteins-2,-3 and -4 in the ovine ovary throughout the oestrous cycle. *J. Endocrinol.* **151** 241-213.

Perrot-Applanat M, Deng M, Fernandez H, Lelaider C, Meduri G, Bouchard P (1994) Immunohistochemical localization of estradiol and progesterone receptors in human uterus throughout pregnancy: expression in endometrial blood vessels. *J. Clin. Endocrinol. Metab.* **78** 216-224.

Pierce JG, Parsons TF (1981) Glycoprotein hormones: structure and function. *Ann. Rev. Biochem.* **50** 465-495.

Polan ML, Seu D, Tarlatzis B (1986) Human chorionic gonadotropin stimulation of estradiol production and androgen antagonism of gonadotropin-stimulated responses in cultured human granulosa-luteal cells. *J. Clin. Endocrinol. Metab.* **62** 628-633.

Poniter CH, Torres BA, Vallet JL, Bazer FW, Johnson HM (1988) Antiviral activity of the pregnancy recognition hormone ovine trophoblastic protein-1. *Biochem. Biophys. Res. Comm.* **152** 801-807.

Powell WS, Hammarström S, Samuelsson B, Sjöberg B (1974) Prostaglandin $F_{2\alpha}$ receptor in human corpora lutea. *Lancet* **i** 1120.

Press MF, Greene GL (1988) Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor. *Endocrinology* **122** 1165-1175.

Press MF, Udove JA, Greene GL (1988) Progesterone receptor distribution in the human endometrium. *Am. J. Pathol.* **131** 112-124.

Priddy AR, Killick SR (1993) Eicosanoids and ovulation. *Prost. Leukotrienes Ess. Fatty Acids* **49** 827-831.

Puistola U, Westerlund A, Kauppila A, Turpeenniemi-Hujanen T (1995) Regulation of 72-kd type IV collagenase-matrix metalloproteinase-2 by estradiol and gonadotrophin-releasing hormone agonist in human granulosa-lutein cells. *Fertil. Steril.* **64** 81-87.

Pullan S, Wilson J, Metcalfe A, Edwards GM, Goberdhan N, Tilly J, Hickman JA, Dive C, Streuli CH (1996) Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. *J. Cell Sci.* **109** 631-642.

Rabinovici J, Spencer SJ, Jaffe RB (1990) Recombinant human activin-A promotes proliferation of human luteinized preovulatory granulosa cells *in vitro*. *J. Clin. Endocrinol. Metab.* **71** 1396-1398.

Rao ChV, Griffin LP, Carman FR Jr (1977a) Prostaglandin $F_{2\alpha}$ binding sites in human corpora lutea. *J. Clin. Endocrinol. Metab.* **44** 1032-1037.

Rao ChV, Griffin LP, Carman FR Jr (1977b) Gonadotropin receptors in human corpora lutea of the menstrual cycle and pregnancy. *Am. J. Obstet. Gynecol.* **128** 146-153.

Rae MT, Menzies GS, McNeilly AS, Woad K, Webb R, Bramley TA (1998) Specific non-genomic, membrane-localized binding sites for progesterone in the bovine corpus luteum. *Biol. Reprod.* **58** 1394-1406.

Rapp G, Freudstein J, Klaudiny J, Mucha J, Wempe F, Zimmer M, Scheit KH (1990) Characterisation of three abundant mRNAs from human ovarian granulosa cells. *DNA Cell Biol.* **9** 479-485.

Ravindranath N, Little-Ihrig LL, Zeleznik AJ (1992a) Characterization of the levels of messenger ribonucleic acid that encode for luteinizing hormone receptor during the luteal phase of the primate menstrual cycle. *J. Clin. Endocrinol. Metab.* **74** 779-785.

Ravindranath N, Little-Ihrig LL, Fairchild Benyo D, Zeleznik AJ (1992b) Role of luteinizing hormone in the expression of cholesterol side-chain cleavage cytochrome P450 and 3β-hydroxysteroid dehydrogenase in the primate corpus luteum. *Endocrinology* **131** 2065-2070.

Raziel A, Ronel R, Pansky M, Arieli S, Bukovsky I, Caspi E (1993) Current management of ruptured corpus luteum. *Eur. J. Obstet. Gynaecol. Reprod. Biol.* **50** 77-81.

Redmer DA, Reynolds LP (1996) Angiogenesis in the ovary. *Rev. Reprod.* **1** 182-192.

Redmer DA, Dai Y, Li J, Charnock-Jones DS, Smith SK, Reynolds LP, Moor RM (1996) Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum. *J. Reprod. Fertil.* **108** 157-165.

Reich R (1991) Preovulatory changes in ovarian expression of collagenases and tissue metalloproteinase inhibitor messenger ribonucleic acid: role of eicosanoids. *Endocrinology* **129** 1869-1875.

Reich R, Tsafriri A, Mechanic GL (1985) Involvement of collagenolysis in ovulation in the rat. *Endocrinology* **116** 522-527.

Retamales I, Carrasco I, Troncoso JL, Las Heras J, Devoto L, Vega M (1994) Morpho-functional study of human luteal cell subpopulations. *Hum. Reprod.* **9** 591-596.

Revelli A, Massobrio M, Tesarik J (1998) Nongenomic actions of steroid hormones in reproductive tisses. *Endocrine Rev.* **19** 3-17.

Reynolds LP, Robertson DA, Ford SP (1983) Effects of intrauterine infusion of oestradiol-17β and prostaglandin E_2 on luteal function in nonpregnant heifers. *J. Reprod. Fertil.* **69** 703-709.

Reynolds LP, Killilea SD, Redmer DA (1992) Angiogenesis in the female reproductive system. *FASEB J.* **6** 886-892.

Richards JS, Midgley AR Jr (1976) Protein hormone action: A key to understanding ovarian follicular and luteal cell development. *Biol. Reprod.* **14** 82-94.

Richards JS, Williams JL (1976) Luteal cell receptor content for prolactin (PRL) and luteinizing hormone (LH): regulation by LH and PRL. *Endocrinology* **99** 1571-1581.

Richards JS, Jahnsen T, Hedin L, Lifka J, Ratoosh SL, Durica JM, Goldring NB (1987) Ovarian follicular development: From physiology to molecular biology. *Rec. Prog. Horm. Res.* **43** 231-270.

Richards JS, Fitzpatrick SL, Clemens JW, Morris JK, Alliston T, Sirois J (1995) Ovarian cell differentiation: a cascade of multiple hormones, cellular signals and regulated genes. *Rec. Prog. Horm. Res.* **50** 223-254.

Richards RG, Almond GW (1994) Identification and distribution of tumor necrosis factor α receptors in pig corpora lutea. *Biol. Reprod.* **51** 1285-1291.

Richardson MC, Masson GM (1985) Lack of direct inhibitory action of oxytocin on progesterone production by dispersed cells from human corpus luteum. *J. Endocrinol.* **104** 149-151.

Ricketts ML, Verheag JM, Bujalska I, Howie AJ, Rainey WE, Stewart PM (1998) Immunohistochemical localization of type I 11β-hydroxysteroid dehydrogenase in human tissues. *J. Clin. Endocrinol. Metab.* **83** 1325-1335.

Riley JCM, Carlson JC (1987) Involvement of phospholipase A activity in the plasma membrane of the rat corpus luteum during luteolysis. *Endocrinology* **121** 776-781.

Riley JCM, Behrman HR (1991) *In vivo* generation of hydrogen peroxide in the rat corpus luteum during luteolysis. *Endocrinology* **128** 1749-1753.

Riley SC, Dupont E, Walton JC, Luu-The V, Labrie F, Pelletier G, Challis JRG (1992) Immunohistochemical localization of 3β-hydroxy-5-ene-steroid dehydrogenase/Δ^5-Δ^4 isomerase in human placenta and fetal membranes throughout gestation. *J. Clin. Endocrinol. Metab.* **75** 956-961.

Ristimaki A, Jaatinen R, Ritvos O (1997) Regulation of prostaglandin $F_{2\alpha}$ receptor expression in cultured human granulosa-luteal cells. *Endocrinology* **138** 191-195.

Rizkallah T, Gurpide E, Vande Wiéle RL (1969) Metabolism of hCG in man. *J. Clin. Endocrinol. Metab.* **29** 92-100.

Roberts AB, Flanders KC, Kondaiah P, Thomson NL, Van Obberghen-Schilling E, Wakefield L, Rossi P, de Crombrugghe B, Heine U, Sporn MB (1988) Transforming growth factor β: biochemistry and roles in embryogenesis, tissue repair and remodeling, and carcinogenesis. *Rec. Prog. Horm. Res.* **44** 157-193.

Roberts JS, McCracken JA, Gavagan JE, Soloff MS (1976) Oxytocin stimulated release of prostaglandin $F_{2\alpha}$ from ovine endometrium *in vitro*: correlation with estrous cycle and oxytocin receptor binding. *Endocrinology* **99** 1107-1114.

Roberts VJ, Barth S, El-Roeiy A, Yen SSC (1993) Expression of inhibin/activin subunits and follistatin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle. *J. Clin. Endocrinol. Metab.* **77** 1402-1410.

Robertson SA, Seamark RF (1990) Granulocyte macrophage colony stimulating factor (GM-CSF) in the murine reproductive tract: stimulation by seminal factors. *Reprod. Fertil. Dev.* **2** 359-368.

Roby KF, Terranova PF (1989) Localization of tumor necrosis factor (TNF) in rat and bovine ovary using immunocytochemistry and cell blot: evidence for granulosal production. In: Hirshfield AN (ed), *Growth Factors and the Ovary*. New York: Plenum Press, pp 273-278.

Rodger FE, Fraser HM, Duncan WC, Illingworth PJ (1995) Immunolocalization of bcl-2 in the human corpus luteum. *Hum. Reprod.* **10** 1566-1570.

Rodger FE, Young FM, Fraser HM, Illingworth PJ (1997) Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic gonadotrophin rescue, in the human corpus luteum. *Hum. Reprod.* **12** 1723-1729.

Rodger FE, Fraser HM, Krajewski S, Reed JC, Illingworth PJ (1998) Expression of the proto-oncogene bax does not vary with changing luteal function in women. *Mol. Hum. Reprod.* **4** 27-32.

Rodgers RJ, O'Shea JD, Findlay JK (1985) Do small and large luteal cells of the sheep interact in the production of progesterone? *J. Reprod. Fertil.* **75** 85-94.

Rojas FJ, Ciridon JW (1996) Evidence that hormone receptors couple and activate a common signal transducer adenylyl cyclase in corpus luteum. *Fertil. Steril.* **65** 275-279.

Rojas FJ, Moretti-Rojas IM, Balmaceda JP, Asch RH (1989) Changes in adenylyl cyclase activity of the human and nonhuman primate corpus luteum during the menstrual cycle and pregnancy. *J. Clin. Endocrinol. Metab.* **68** 379-385.

Rothchild I (1981) The regulation of the mammalian corpus luteum. *Rec. Prog. Horm. Res.* **37** 183-298.

Rothchild I (1996) The corpus luteum revisited: are the paradoxical effects of RU486 a clue to how progesterone stimulates its own secretion? *Biol. Reprod.* **55** 1-4.

Rueda BR, Wegner JA, Marion SL, Wahlen DD, Hoyer PB (1995a) Internucleosomal DNA fragmentation in ovine luteal tissue associated with luteolysis: *in vivo* and *in vitro* analysis. *Biol. Reprod.* **52** 305-312.

Rueda BR, Botros IW, Pierce KL, Regan JW, Hoyer PB (1995b) Comparison of messenger RNA levels for the $PGF_{2\alpha}$ receptor (FP) during luteolysis and early pregnancy in the ovine corpus luteum. *Endocrine* **3** 781-787.

Russell DL, Salamonsen LA, Findlay JK (1995) Immunization against the N-terminal peptide of the inhibin α43-subunit (αN) disrupts tissue remodeling and the increase in matrix metalloproteinase-2 during ovulation. *Endocrinology* **136** 2657-3664.

Salamonsen LA (1996) Matrix metalloproteinases and their tissue inhibitors in endocrinology. *Trends Endocrinol. Metab.* **7** 28-34.

Salamonsen LA, Woolley DE (1996) Matrix metalloproteinases and their tissue inhibitors in endometrial remodelling and menstruation. *Reprod. Med. Rev.* **5** 185-203.

Salim A, Zalud I, Farmakides G, Schulman H, Kurjak A, Latin V (1994) Corpus luteum blood flow in normal and abnormal early pregnancy. *J. Ultrasound Med.* **13** 971-975.

Samuelsson B, Granstrom E, Green K, Hamberg M (1971) Metabolism of prostaglandins. *Ann. NY Acad. Sci.* **180** 138-161.

San Roman GA, Magoffin DA (1992) Insulin-like growth factor binding proteins in ovarian follicles from women with polycystic ovarian disease: Cellular source and levels in follicular fluid. *J. Clin. Endocrinol. Metab.* **75** 1010-1016.

Sanchez-Criado JE, Ochiai K, Rothchild I (1987) Indomethacin treatment prevents prolactin-induced luteolysis in the rat. *J. Endocrinol.* **112** 317-322.

Sanders SL, Stouffer RL (1997) Localization of steroidogenic enzymes in macaque luteal tissue during the menstrual cycle and simulated early pregnancy: immunohistochemical evidence supporting the two-cell model for estrogen production in the primate corpus luteum. *Biol. Reprod.* **56** 1077-1087.

Sargent EL, Baughman WL, Novy MJ, Stouffer RL (1988) Intraluteal infusion of a prostaglandin synthesis inhibitor, sodium meclofenamate, causes premature luteolysis in rhesus monkeys. *Endocrinology* **123** 2261-2269.

Sasano H, Okamoto M, Mason JI, Simpson ER, Mendelson CR, Sasano N, Silverberg SG (1989) Immunolocalization of aromatase, 17α-hydroxylase, and side-chain-cleavage cytochromes P-450 in the human ovary. *J. Reprod. Fertil.* **85** 163-169.

Sawada M, Carlson JC (1991) Rapid plasma membrane changes in superoxide radical formation, fluidity, and phospholipase A_2 activity in the corpus luteum of the rat during induced luteolysis. *Endocrinology* **128** 2992-2998.

Sawyer HR, Niswender KD, Braden TD, Niswender GD (1990) Nuclear changes in ovine luteal cells in response to $PGF_{2\alpha}$. *Domest. Anim. Endocrinol.* **7** 229-238.

Schaison G, George M, Lestrat N, Reinberg A, Baulieu EE (1985) Effects of the antiprogesterone steroid RU486 during midluteal phase in normal women. *J. Clin. Endocrinol. Metab.* **61** 484-486.

Schoonmaker JN, Bergman KS, Steiner RA, Karsch FJ (1982) Estradiol-induced luteal regression in the rhesus monkey: evidence for an extra-ovarian site of action. *Endocrinology* **110** 1708-1715.

Schulz KD, Geiger W, Del Pozo E, Kunzig HJ (1978) Pattern of sexual steroids, prolactin and gonadotropic hormones during prolactin inhibition in normally cycling women. *Am. J. Obstet. Gynecol.* **132** 561-566.

Segaloff DL, Ascoli M (1993) The lutropin/choriogonadotropin receptor... 4 years later. *Endocrine Rev.* **14** 324-347.

Senturk LM, Mor G, Gutierrez LS, Zeyneloglu HB, Bahtiyar MO, Arici A (1997) Monocyte chemoattractant protein-1 expression in human corpus luteum. *Fertil. Steril.* **SS** 170.

Sheldrick EL, Flint APF (1985) Endocrine control of uterine oxytocin receptors in the ewe. *J. Endocrinol.* 106 249-258.

Sheldrick EL, Mitchell MD, Flint APF (1980) Delayed luteal regression in ewes immunized against oxytocin. *J. Reprod. Fertil.* **59** 37-42.

Shemesh MF, Milaguir F, Ayalon N, Hansel W (1979) Steroidogenesis and prostaglandin synthesis by cultured bovine blastocysts. *J. Reprod. Fertil.* **56** 181-185.

Sherwood OD (1994) Relaxin. In: Knobil E, Neill JD (eds), *The Physiology of Reproduction*, Second edition. New York: Raven Press, pp861-1009.

Shi S, Chaiwun B, Young I, Cote RJ, Taylor CR (1993) Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *J. Histochem. Cytochem.* **41** 1599-1604.

Shiels RM, Duncan WC, Illingworth PJ (1996) Expression and immunolocalisation of nitric oxide synthase isoforms in the human corpus luteum. *J. Reprod. Fertil.* **17** (Abstract Series) 92.

Shikone T, Yamoto M, Kokawa K, Yamashita K, Nishimori K, Nakano R (1996) Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy. *J. Clin. Endocrinol. Metab.* **81** 2376-2380.

Shima DT, Saunders KB, Gougos A, Damore PA (1995) Alterations in gene expression associated with changes in the state of endothelial differentiation. *Differentiation* **58** 217-226.

Shima K, Kitayama S, Nakano R (1987) Gonadotropin binding sites in human ovarian follicles and corpora lutea during the menstrual cycle. *Obstet. Gynecol.* **69** 800-806.

Shinozaki H, Ito I, Hasegawa Y, Nakamura K, Igarashi S, Nakamura M, Miyamoto K, Eto Y, Ibuki Y, Minegishi T (1992) Cloning and sequencing of a rat type II activin receptor. *FEBS Lett.* **312** 53-56.

Shutt DA, Clark AH, Fraser IS, Goh P, McMahon GR, Saunders DM, Shearman RP (1976) Changes in concentration of prostaglandin F and steroids in human corpora lutea in relation to growth of the corpus luteum and luteolysis. *J. Endocrinol.* **71** 453-454.

Simon AM, Goodenough DA, Li E, Paul DL (1997) Female infertility in mice lacking connexin 37. *Nature* **385** 525-529.

Simpson ER, Boyd GS (1967) The cholesterol side-chain cleavage system of the adrenal cortex. *J. Biochem.* **2** 275-285.

Sjögren A, Holmes PV, Hillensjo T (1991) Interleukin-1α modulates luteinizing hormone stimulated cyclic AMP and progesterone release from human granulosa cells *in vitro*. *Hum. Reprod.* **6** 910-913.

Slayden OD, Zelinski-Wooten MB, Stouffer RL, Brenner RM (1994) Radioligand binding assay of progesterone receptors in the primate corpus luteum after *in vivo* treatment with the 3β-hydroxysteroid dehydrogenase inhibitor, trilostane. *J. Clin. Endocrinol. Metab.* **79** 620-626.

Smith GW, Moor RM. Smith MF (1993) Identification of a 30 000 M_r polypeptide secreted by cultured ovine granulosa cells and luteal tissue as a tissue inhibitor of metalloproteinases. *Biol. Reprod.* **48** 125-132.

Smith GW, Goetz TL, Anthony RV, Smith MF (1994) Molecular cloning of an ovine ovarian tissue inhibitor of metalloproteinases: ontogeny of messenger ribonucleic acid expression and *in situ* localization within preovulatory follicles and luteal tissue. *Endocrinology* **134** 344-352.

Smith GW, McCrone S, Petersen SL, Smith MF (1995a) Expression of messenger ribonucleic acid encoding tissue inhibitor of metalloproteinases-2 within ovine follicles and corpora lutea. *Endocrinology* **136** 570-576.

Smith GW, Gentry PC, Long DK, Bao B, Roberts RM, Smith MF (1995b) Expression of progesterone receptor (P_4R) messenger RNA within ovine post-surge follicles and corpora lutea. *Biol. Reprod.* **52** (Suppl. 1) 67.

Smith GW, Gentry PC, Roberts RM, Smith MF (1996a) Ontogeny and regulation of luteinizing hormone receptor messenger ribonucleic acid within the ovine corpus luteum. *Biol. Reprod.* **54** 76-83.

Smith GW, Juengel JL, McIntush EW, Youngquist RS, Garverick HA, Smith MF (1996b) Ontogenies of messenger RNA encoding tissue inhibitor of metalloproteinases 1 and 2 within bovine periovulatory follicles and luteal tissue. *Dom. Animal. Endocrinol.* **13** 151-160.

Smith GW, Gentry PC, Bao B, Long DK, Roberts RM, Smith MF (1997) Control of the extracellular matrix remodelling within ovarian tissues: localization and regulation of gene expression of plasminogen activator inhibitor type-1 within the ovine corpus luteum. *J. Reprod. Fertil.* **110** 107-114.

Smith KB, Lunn SF, Fraser HM (1990) Inhibin secretion during the ovulatory cycle and pregnancy in the common marmoset monkey. *J. Endocrinol.* **126** 489-495.

Smith KB, Millar MR, McNeilly AS, Illingworth PJ, Fraser HM, Baird DT (1991) Immunocytochemical localization of inhibin α-subunit in the human corpus luteum. *J. Endocrinol.* **129** 155-160.

Smith MF, Kemper CN, Smith GW, Goetz TL, Jarrell VL (1994) Production of tissue inhibitor of metalloproteinases-1 by porcine follicular and luteal cells. *J. Animal Sci.* **72** 1004-1012.

Smith MS, Freeman ME, Neill JD (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* **96** 219-226.

Soto E, Silavin SL, Tureck RW, Strauss JF III (1984) Stimulation of progesterone synthesis in luteinized human granulosa cells by human chorionic gonadotrophin and 8-bromo-adenosine 3',5'-monophosphate: the effect of low density lipoproteins. *J. Clin. Endocrinol. Metab.* **58** 831-837.

Spanel-Borowski K (1991) Diversity of ultrastructure in different phenotypes of cultures microvessel endothelial cells isolated from bovine corpus luteum. *Cell Tissue Res.* **266** 37-49.

Spanel-Borowski K, Ricken AM (1997) Evidence for the maintenance of macrophage-like cells in long-term bovine granulosa cell culture. *Cell Tissue Res.* **288** 529-538.

Spencer SJ, Cataldo NA, Jaffe RB (1996) Apoptosis in the human female reproductive tract. *Obstet. Gynecol. Survey* **51** 314-323.

Spies HG, Niswender GD (1971) Levels of prolactin, LH and FSH in the serum of intact and pelvic-neuroectomized rats. *Endocrinology* **88** 937-943.

Stamouli A, O'Sullivan MJB, Frankel S, Thomas EJ, Richardson MC (1996) Suppression of matrix metalloproteinase production by hCG in cultures of human luteinized granulosa cells as a model for gonadotrophin-induced luteal rescue. *J. Reprod. Fertil.* **105** 235-239.

Stetler-Stevenson WG, Brown PD, Onisto M, Levy AT, Liotta LA (1990) Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell line and human tumor tissues. *J. Biol. Chem.* **265** 13933-13938.

Stocco DM, Clark BJ (1996) Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Rev.* **17** 221-244.

Stoelk E, Chegini N, Lei ZM, Rao CHV, Bryant-Greenwood G, Sanfilippo J (1991) Immunocytochemical localization of relaxin in human corpora lutea: Cellular and subcellular distribution and dependence on reproductive state. *Biol. Reprod.* **44** 1140-1147.

Stouffer RL (1988) Perspectives on the corpus luteum of the menstrual cycle and early pregnancy. *Sem. Reprod. Endocrinol.* **6** 103-113.

Stouffer RL (1990) Corpus luteum function and dysfunction. *Clin. Obstet. Gynecol.* **33** 668-689.

Stouffer RL, Hodgen GD (1980) Induction of luteal phase defects in rhesus monkeys by follicular fluid administration at the onset of the menstrual cycle. *J. Clin. Endocrinol. Metab.* **51** 669-671.

Stouffer RL, Gulyas BJ, Nixon WE, Hodgen GD (1977) Gonadotropin-sensitive progesterone production by rhesus monkey luteal cells *in vitro*: a function of the age of the corpus luteum during the menstrual cycle. *Endocrinology* **100** 506-512.

Stouffer RL, Coensgen JL, Hodgen GD (1980) Progesterone production by luteal cells isolated from cynomolgus monkeys: effect of gonadotropin and prolactin during acute incubation and cell culture. *Steroids* **35** 523-532.

Stouffer RL, Ottobre JS, VandeVoort CA (1987) Regulation of the primate corpus luteum during early pregnancy. In: Stouffer RL (ed), *The Primate Ovary*, Serono Symposia. New York, Plenium Publishing, pp207-220.

Strauss JF III, Miller WL (1991) Molecular basis of ovarian steroid synthesis. In: Hillier SG (ed), *Ovarian Endocrinology*. Oxford: Blackwell Scientific Publications, pp25-72.

Strauss JF III, Schuler LA, Rosenblum MF, Tanaka T (1981) Cholesterol metabolism by ovarian tissue. *Adv. Lipid Res.* **18** 99-157.

Sugawara T, Holt JA, Driscoll D, Strauss JF III, Lin D, Miller WL, Patterson D, Clancy KP, Hart IM, Clark BJ, Stocco DM (1995) Human steroidogenic acute regulatory protein: functional activity in COS-1 cells, tissue specific expression, and mapping of the structural gene to 8p11.2 and a pseudogene to chromosome 13. *Proc. Natl. Acad. Sci. USA* **92** 4778-4782.

Sugino N, Telleria CM, Gibori G (1997) Progesterone inhibits 20α-hydroxysteroid dehydrogenase expression in the rat corpus luteum through the glucocorticoid receptor. *Endocrinology* **138** 4497-4500.

Suzuki T, Sasano H, Tamura M, Aoki H, Fukaya T, Yajima A, Nagura H, Mason JI (1993) Temporal and spacial localization of steroidogenic enzymes in premenopausal human ovaries: *in situ* hybridization and immunohistochemical study. *Mol. Cell. Endocrinol.* **97** 135-143.

Suzuki T, Sasano H, Kimura N, Tamura M, Fukaya T, Yajima A, Nagura H (1994) Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: relationship to expression of steroidogenic enzymes. *Hum. Reprod.* **9** 1589-1595.

Swanston IA, McNatty KP, Baird DT (1977) Concentrations of prostaglandin F and steroids in the human corpus luteum. *J. Endocrinol.* **73** 115-122.

Tadakuma H, Okamora H, Kitaoka M, Iyama K, Usuku G (1993) Association of immunolocalization of matrix metalloproteinase 1 with ovulation in hCG-treated rat ovary. *J. Reprod. Fertil.* **98** 1869-1875.

Takao Y, Honda T, Ueda M, Hattori N, Yamada S, Maeda M, Fujiwara H, Mori T, Wimalasena J (1997) Immunohistochemical localization of the LH/hCG receptor in human ovary: hCG enhances cell surface expression of LH/hCG receptor on luteinizing granulosa cells *in vitro*. *Mol. Hum. Reprod.* **3** 569-578.

Takaya R, Fukaya T, Sasano H, Suzuki T, Tamura M, Yajima A (1997) Macrophages in normal cycling human ovaries; immunohistochemical localization and characterization. *Hum. Reprod.* **12** 1508-1512.

Talavera F, Menon KM (1989) Regulation of rat luteal cell high density lipoprotein receptors: up regulation in response to changes in intracellular cholesterol concentration. *Endocrinology* **125** 2015-2021.

Tamura M, Sasano H, Suzuki T, Fukaya T, Funayama Y, Takayama K, Takaya R, Yajima A (1995) Expression of epidermal growth factors and

epidermal growth factor receptor in normal cycling human ovaries. *Hum. Reprod.* **10** 1891-1896.

Tamura T, Kitawaki J, Yamamoto T, Osawa Y, Kominami S, Takemori S, Okada H (1992) Immunohistochemical localization of 17-α hydroxylase/ C17-20 lyase and aromatase cytochrome P-450 in the human ovary during the menstrual cycle. *J. Endocrinol.* **135** 589-595.

Tan GJS, Biggs JSG (1983) Effects of prolactin on steroid production by human luteal cells *in vitro*. *J. Endocrinol.* **96** 499-503.

Tan GJS, Tweedale R, Biggs JSG (1982a) Oxytocin may play a role in the control of the corpus luteum. *J. Endocrinol.* **95** 65-70.

Tan GJS, Tweedale R, Biggs JSG (1982b) Effects of oxytocin on the bovine corpus luteum of early pregnancy. *J. Reprod. Fertil.* **66** 75-78.

Thau RB, Yamamoto Y, Sundaram K, Spinola PG (1983) Human chorionic gonadotropin stimulates luteal function in rhesus monkeys immunized against the β subunit of ovine luteinizing hormone. *Endocrinology* **112** 277-283.

Themmen APN, Kraaij R, Grootegoed JA (1994) Regulation of gonadotropin receptor gene expression. *Mol. Cell. Endocrinol.* **100** 15-19.

Tilly JL (1996) Apoptosis and ovarian function. *Rev. Reprod.* **1** 162-172.

Tinkanen H (1994) The role of vascularization of the corpus luteum in the short luteal phase studied by doppler ultrasound. *Acta Obstet. Gynecol. Scand.* 73 321-323.

Toaff R, Toaff ME, Peyser MR (1976) Infertility following wedge resection of the ovaries. *Am. J. Obstet. Gynecol.* **124** 92-96.

Torii R, Abbott DH, Nigi H (1996) Morphological changes of the ovary and hormonal changes through the ovarian cycle of the common marmoset (*Callithrix jacchus*). *Primates* **37** 49-56.

Toth P, Li X, Rao ChV, Lincoln SR, Sanfilippo JS, Spinnato JA II, Yussman MA (1994) Expression of functional human chorionic gonadotropin/human

luteinizing hormone receptor gene in human uterine arteries. *J. Clin. Endocrinol. Metab.* **79** 307-315.

Tovanabutra S, Illingworth PJ, Ledger WL, Glasier AF, Baird DT (1993) The relationship between peripheral immunoactive inhibin, human chorionic gonadotrophin, oestradiol and progesterone during human pregnancy. *Clin. Endocrinol.* **38** 101-107.

Townson DH, Warren JS, Flory CM, Naftalin DM, Keyes PL (1996) Expression of monocyte chemoattractant protein-1 in the corpus luteum of the rat. *Biol. Reprod.* **54** 513-520.

Tsafriri A, Adashi EY (1994) Local nonsteroidal regulators of ovarian function. In: Knobil E, Neill JD (eds), *The Physiology of Reproduction*, Second edition. New York: Raven Press, pp817-860.

Tsafriri A, Braw RH (1984) Experimental approaches to atresia in mammals. *Oxf. Rev. Reprod. Biol.* **6** 226-265.

Tsafriri A, Dekel N (1994) Molecular mechanisms in ovulation. In: Findlay J.K. (ed), *Molecular Biology of the Female Reproductive System.* London: Academic Press, pp207-258.

Tsang PCW, Poff JP, Boulton EP, Condon WA (1995) Four-day-old bovine corpus luteum: progesterone production and identification of matrix metalloproteinase activity *in vitro*. *Biol. Reprod.* **53** 1160-1168.

Tulchinsky D, Hobel CJ (1973) Plasma human chorionic gonadotropin, estrone, estradiol, progesterone and 17α hydroxyprogesterone in human pregnancy. III. Early normal pregnancy. *Am. J. Obstet. Gynecol.* **113** 751-758.

Tung L, Mohamed MK, Hoeffler JP, Takimoto GS, Horwitz KB (1993) Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors. *Mol. Endocrinol.* **7** 1256-1265.

Turner KJ, McKinnell C, McLaren TT, Qureshi SJ, Saunders PTK, Foster PMD, Sharpe RM (1996) Detection of germ cell-derived proteins in testicular interstitial fluid - potential for monitoring spermatogenesis. *J. Androl.* **17** 127-136.

Ulisse S, Farina AR, Piersanti D, Tiberio A, Cappabianca I, Dorazi G, Jannini EA, Malykh O, Stetler-Stevenson WG, Darmiento M, Gulino A, Mackay AR (1994) Follicle-stimulating hormone increases the expression of tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 and induces TIMP-1 AP-1 site binding complex(es) in prepubertal rat sertoli cells. *Endocrinology* **135** 2479-2487.

Uria JA, Ferrando AA, Velasco G, Freije JMP, Lopez-Otin C (1994) Structure and expression in breast tumors of human TIMP-3, a new member of the metalloproteinase inhibitor family. *Cancer Res.* **54** 2091-2094.

Van Voorhis BJ, Dunn MS, Snyder GD, Weiner CP (1994) Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* **135** 1799-1805.

Vandessel HJHMT, Chandrasekher Y, Yap OWS, Lee PDK, Hintz RL, Faessen GHJ, Brat DDM, Fauser BCJM, Giudice LC (1996) Serum and follicular fluid levels of insulin growth factor-I (IGF-I), IGF-II, and IGF binding protein-1 and IGF binding protein-3 during the normal menstrual cycle. *J. Clin. Endocrinol. Metab.* **81** 1224-1231.

Vega M, Carrasco I, Castillo T, Troncoso JL, Videla LA, Devoto L (1995) Functional luteolysis in response to hydrogen peroxide in human luteal cells. *J. Endocrinol.* **147** 177-182.

Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP (1993) Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol. Endocrinol.* **7** 1244-1255.

Veldhuis JD, Garmey JC, Urban RJ, Demers LM, Aggarwal BB (1991) Ovarian actions of tumor necrosis factor-α (TNFα): pleiotropic effects of TNFα on differentiated functions of untransformed swine granulosa cells. *Endocrinology* **129** 641-648.

von Berswoldt-Wallabre I, Geller HF, Herlyn U (1964) Temporal aspects of decidual cell reaction. I. Induction of decidual cell reaction in lactogenic hormone treated and in pseudopregnant rats. *Acta Endocrinol.* **45** 349-352.

Voultilainen R, Tapanainen J, Chung B-C, Matteson KJ, Miller WL (1986) Hormonal regulation of $P450_{scc}$ (20,22-desmolase) and $P450_{c17}$ (17α-hydroxylase/17,20-lyase) in cultured human granulosa cells. *J. Clin. Endocrinol. Metab.* **63** 202-207.

VuHai-LuuThi MT, Misrahi M, Houllier A, Jolivet A, Milgrom E (1992) Variant forms of the pig lutropin/choriogonadotropin receptor. *Biochemistry* **31** 8377-8383.

Wada M, Shintani Y, Kosaka M, Sano T, Hizawa K, Saito S (1996) Immunohistochemical localization of activin-A and follistatin in human tissues. *Endocrine J.* **43** 375-385

Waddell BJ, Benediktsson R, Seckl JR (1996) 11β-hydroxysteroid dehydrogenase type-2 in the rat corpus luteum - induction of messenger ribonucleic acid expression and bioactivity coincident with luteal regression. *Endocrinology* **137** 5386-5391.

Wang F, Riley JCM, Berhman HR (1993) Immunosuppressive glucocorticoid blocks extrauterine luteolysins in the rat. *Biol. Reprod.* **49** 66-75.

Wang LJ, Robertson S, Seamark RF, Norman RJ (1991) Lymphokines including interleukin-2, alter gonadotropin-stimulated progesterone production and proliferation of human granulosa-lutein cells *in vitro*. *J. Clin. Endocrinol. Metab.* **72** 824-831.

Wang LJ, Pascoe V, Petrucco OM, Norman RJ (1992) Distribution of leukocyte subpopulations in the human corpus luteum. *Hum. Reprod.* **7** 197-202.

Wang HZ, Lu SH, Han XJ, Zhou W, Sheng WX, Sun ZD, Gong YT (1992) Inhibitory effect of interferon and tumor necrosis factor on human luteal function *in vitro*. *Fertil. Steril.* **58** 941-945.

Wang QF, Tilly KI, Tilly JL, Preffer F, Schneyer AL, Crowley WF, Sluss PM (1996) Activin inhibits basal and androgen-stimulated proliferation and induces apoptosis in the human prostatic cancer cell line LNCaP. *Endocrinology* **137** 5476-5483.

Waterman MR (1995) A rising StAR: an essential role in cholesterol transport. *Science* **267** 1780-1781.

Wathes DC, Swann RW (1982) Is oxytocin an ovarian hormone? *Nature* 297 225-227.

Webley GE, Richardson MC, Smith CA, Masson GM, Hearn JP (1990) Size distribution of luteal cells from pregnant and nonpregnant marmoset monkeys and a comparison of the morphology of marmoset luteal cells with those from the human corpus luteum. *J. Reprod. Fertil.* **90** 427-437.

Webley GE, Hodges JK, Given A, Hearn JP (1991) Comparison of the luteolytic action of gonadotropin-releasing hormone antagonist and cloprostenol, and the ability of human chorionic gonadotropin and melatonin to override their luteolytic effects in the marmoset monkey. *J. Endocrinol.* **128** 121-129.

Wei LL, Gonzalez-Aller C, Wood WM, Miller LA, Horwitz KB (1990) 5'-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. *Mol. Endocrinol.* **4** 1833-1840.

Weinstein D, Polishuk W (1975) The role of wedge resection of the ovary as a cause of mechanical sterility. *Surg. Obstet. Gynecol.* **141** 417-418.

Weiss TJ, Seamark RF, McIntosh JEA, Moor RM (1976) Cyclic AMP in sheep ovarian follicles: site of production and response to gonadotrophins. *J. Reprod. Fertil.* **46** 347-353.

Wentz AC, Jones GS (1973) Transient luteolytic effect of prostaglandin $F_{2\alpha}$ in the human. *Obstet. Gynecol.* **42** 172-181.

Wilcox LS, Chu SY, Eaker ED, Zeger SL, Peterson HB (1991) Risk factors for regret after tubal sterilization - 5 years of follow up in a prospective study. *Fertil. Steril.* **55** 927-933.

Wilks JW (1980) Effect of (15S)-15-methyl-prostaglandin $F_{2\alpha}$ methyl ester and estrogens upon the corpus luteum and conceptus of the rhesus monkey. *Prostaglandins* **20** 807-823.

Wilson CA, Leigh AJ, Chapman AJ (1990) Gonadotrophin glycosylation and function. *J. Endocrinol.* **125** 3-14.

Wiltbank MC, Belfiore CJ, Niswender GD (1993) Steroidogenic enzyme activity after acute activation of protein kinase (PK) A and PKC in ovine small and large luteal cells. *Mol. Cell. Endocrinol.* **97** 1-7.

Woad KJ, Norman B, Fraser HM (1996) Immunohistochemical localisation of clusterin during luteolysis in the primate. *J. Reprod. Fertil.* **17** (Abstract series) 59.

Woessner JF Jr (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* **5** 2145-2154.

Wolf V, Ke G, Dharmarajan AM, Bielke W, Artuso L, Saurer S, Friis R (1997) DDC-4, an apoptosis-associated gene is a secreted frizzled relative. *FEBS Lett.* **417** 385-389.

Woodruff TK, Krummen L, McCray G, Mather JP (1993) *In situ* ligand-binding of recombinant human I^{125} activin A and recombinant I^{125} inhibin A to the adult rat ovary. *Endocrinology* **133** 2998-3006.

Wuttke W, Jarry H, Pitzel L, Knoke I, Spiess S (1993) Luteotrophic and luteolytic actions of ovarian peptides. *Hum. Reprod.* **8** (Suppl. 2) 141-146.

Wyllie AH (1994) Death from inside out: an overview. *Phil. Trans. R. Soc. Lond. B* **345** 237-241.

Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68** 251-306.

Yamoto M, Nishimori K, Nakana R (1988) Masked gonadotropin-binding sites in human corpora lutea during menstrual cycle and pregnancy. *Fertil. Steril.* **50** 239-244.

Yeko TR, Khan-Dawood FS, Dawood MY (1989) Human corpus luteum: luteinizing hormone and chorionic gonadotropin receptors during the menstrual cycle. *J. Clin. Endocrinol. Metab.* **68** 529-534.

Yen SSC (1986) The human menstrual cycle. In: Yen SSC, Jaffe RB (eds), *Reproductive Endocrinology*, Second edition. New York: Saunders pp33-74.

Ying SY (1988) Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrine Rev.* **9** 267-293.

Yoshimura Y, Nakamura Y, Ichikawa F, Oda T, Jinno M, Ando M, Koyama N, Shiokawa S (1992) Possible involvement of leukotrienes in human luteal function. *Acta Endocrinol.* **127** 246-251.

Young FM, Illingworth PJ, Lunn SF, Harrison DJ, Fraser HM (1997) Cell death during luteal regression in the marmoset monkey (*Callithrix jacchus*). *J. Reprod. Fertil.* **111** 109-119.

Yuan W, Giudice LC (1997) Programmed cell death in human ovary is a function of follicle and corpus luteum status. *J. Clin. Endocrinol. Metab.* **82** 3148-3155.

Zeleznik AJ, Hillier SG (1996) The ovary: endocrine function. In: Hillier SG, Kitchener HC, Neilson JP (eds), *Scientific Essentials of Reproductive Medicine.* London: WB Saunders, pp133-146.

Zeleznik AJ, Little-Ihrig LL (1990) Effect of reduced luteinizing hormone concentrations on corpus luteum function during the menstrual cycle of rhesus monkeys. *Endocrinology* **126** 2237-2244.

Zeleznik AJ, Fairchild Benyo D (1994) Control of follicular development, corpus luteum function, and the recognition of pregnancy in higher primates. In: Knobil E, Neill JD (eds), *The Physiology of Reproduction*, Second edition. New York: Raven Press, pp751-780.

Zelinski-Wooten MB, Stouffer RL (1990) Intraluteal infusions of prostaglandins of the E, D, I, and A series prevent $PGF_{2\alpha}$-induced, but not spontaneous, luteal regression in rhesus monkeys. *Biol. Reprod.* **43** 507-516.

Zhang FP, Rannikko AS, Manna PR, Fraser HM, Huhtaniemi IT (1997) Cloning and functional expression of the luteinizing hormone receptor complementary deoxyribonucleic acid from the marmoset monkey testes: absence of sequences encoding exon 10 in other species. *Endocrinology* **138** 2481-2490.

Zhao Y, Luck MR (1996) Bovine granulosa cells express extracellular matrix proteins and their regulators during luteinization in culture. *Reprod. Fertil. Dev.* **8** 259-266.

Zhao Y, Rong H, Chegini N (1995) Expression and selective cellular localization of granulocyte-macrophage colony-stimulating factor (GM-CSF) and GM-CSF α and β receptor messenger ribonucleic acid and protein in human ovarian tissue. *Biol. Reprod.* **53** 923-930.

Zheng J, Fricke PM, Reynolds LP, Redmer DA (1994) Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. *Biol. Reprod.* **51** 623-632.

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Luteinizing hormone receptor in the human corpus luteum: lack of down-regulation during maternal recognition of pregnancy

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Luteal progesterone production is dependent on luteinizing hormone (LH) from the pituitary gland. Despite continuing LH secretion, the human corpus luteum undergoes functional luteolysis unless it is 'rescued' by human chorionic gonadotrophin (HCG), produced by the implanting blastocyst. As LH and HCG act through a common receptor, this study sought to determine the expression of the LH/HCG receptor in the corpus luteum during maternal recognition of pregnancy. Corpora lutea were collected at hysterectomy from women in the normal luteal phase and after luteal 'rescue' with exogenous HCG. In each case the corpus luteum was classified according to the date of the LH surge measured in daily urine samples. The expression of the LH receptor was investigated by Northern blotting, in-situ hybridization and in-situ ligand binding. LH receptor mRNA and ligand binding activity were detected in corpora lutea from all stages of the luteal phase. LH receptor expression and binding were maintained during maternal recognition of pregnancy in the presence of exponentially increasing HCG concentrations. These data show that the LH receptor is maintained throughout the functional life-span of the human corpus luteum and is not down-regulated during maternal recognition of pregnancy.

Key words: corpus luteum/human chorionic gonadotrophin/luteinizing hormone/pregnancy/receptor

Introduction

Progesterone production by the primate corpus luteum is dependent on circulating luteinizing hormone (LH) from the pituitary gland (Hutchison and Zeleznik, 1984; Fraser *et al.*, 1986). Withdrawal of circulating LH results in both structural and functional luteolysis. However, despite the continued secretion of LH, functional luteolysis occurs after 14 days unless human chorionic gonadotrophin (HCG) is secreted by the implanting blastocyst (Hutchison *et al.*, 1986). Both LH and HCG exert their luteotrophic actions through a common receptor (Cole *et al.*, 1973). The human LH/HCG receptor cDNA has now been cloned and sequenced (Minegish *et al.*, 1990). It is part of a family of G-protein-coupled receptors, with seven transmembrane regions and a large glycosylated extracellular domain (Segaloff and Ascoli, 1993).

In the presence of increasing HCG, during maternal recognition of pregnancy in women, luteal progesterone production increases and circulating progesterone concentrations rise (Lenton and Woodward, 1988; Tovanabutra *et al.*, 1993). However, in other species the LH receptor has been shown to undergo desensitization and down-regulation after exposure to its ligand (Niswender *et al.*, 1985; Segaloff and Ascoli, 1993; Peegel *et al.*, 1994). In addition, previous studies investigating the LH receptor in the human corpus luteum have reported low levels of receptor in the corpus luteum of ectopic pregnancy (Rao *et al.*, 1977; Bramley *et al.*, 1987; Yamoto *et al.*, 1988). It is therefore not clear how the human corpus luteum is able to augment progesterone production during maternal recognition of pregnancy.

This study aimed to investigate the luteal LH/HCG receptor during the process of maternal recognition of pregnancy at the time of menstrual delay. We studied LH/HCG receptor mRNA expression and ligand binding in carefully dated human corpora lutea from throughout the normal luteal phase and after luteal 'rescue' with logarithmically increasing doses of exogenous HCG.

Materials and methods

Source of reagents

All reagents were obtained from Sigma Chemical Company (Poole, UK), unless otherwise stated. A 1.5 kb cDNA construct, corresponding to nucleotide 542 to the last nucleotide of the open reading frame (2124) of the human LH receptor in pBluescript (Stratagene, Cambridge, UK), was kindly supplied by Dr M.Atger of the Faculté de Médècine de Bicêtre, Université Paris-Sud, Le Kremlin-Bicêtre, France. ^{125}I-labelled LH was obtained commercially from the Department of Chemical Pathology, Hammersmith Hospital, London, UK. The specific activity of the [^{125}I]LH was 100 μCi/μg, and 10 000 c.p.m. is equivalent to 45 pg.

Collection of tissue

Corpora lutea were enucleated at the time of hysterectomy in women undergoing surgery for benign conditions, typically heavy and/or painful menses. All women were healthy, aged 32–45 years, with regular menstrual cycles and had not received any form of hormonal treatment for at least 3 months prior to taking part in the study. The date of the LH surge was determined by estimating the LH concentrations in serial early morning urine samples collected prior to the operation (Djahanbakhch *et al.*, 1981a). On this basis, four corpora lutea were classified as early luteal (LH+1 to LH+5), four as mid-luteal (LH+6 to LH+10) and four as late luteal (LH+11 to LH+14). In addition, four women were given i.m. injections of HCG

(Profasi; Serono Laboratories, Welwyn Garden City, UK) from day LH+7 in daily doubling doses, starting at 125 IU, for 5–8 days until surgery. This regimen has been shown to reproduce the hormonal changes of early pregnancy (Illingworth *et al.*, 1990).

As described previously (Duncan *et al.*, 1996), the whole corpus luteum was enucleated from the ovary by blunt dissection and the ovary oversewn. The tissue was divided immediately into radial blocks to ensure that the whole thickness of the gland was represented in any piece. A piece of tissue was rapidly snap frozen in liquid nitrogen and stored at –70°C for subsequent RNA extraction. Another piece of the biopsy was frozen in embedding medium (Tissue-Tek OCT compound; Miles Inc., Elkhart, IN, USA) and stored at –70°C until frozen sections were cut. Frozen sections were stored at –70°C until required. In each case, an endometrial biopsy was also fixed in 4% paraformaldehyde and processed into paraffin wax for luteal phase dating by tissue morphometry (Li *et al.*, 1988). Blood was taken before surgery and the plasma progesterone concentration was measured by a standard radioimmunoassay (Djahanbakhch *et al.*, 1981b). This study was approved by the local Reproductive Medicine Ethics Committee, and informed consent was obtained from all patients prior to tissue collection.

Northern blotting

Total RNA was isolated by the method of Chomczynski and Sacchi (1987) using a commercial kit. Its concentration was determined by absorption at 260 nm. Total RNA (25 µg) was denatured, electrophoresed in a 1.5% formaldehyde–agarose gel and transferred to a nylon membrane (Amersham International plc, Aylesbury, UK) by capillary action in 20× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). The RNA was fixed onto the membranes by UV cross-linkage (Spectronics Corporation, New York, NY, USA). Membranes were prehybridized for 3 h in 15 ml hybridization buffer [0.5 M sodium phosphate, 1 mM EDTA, 1% (w/v) bovine serum albumin (BSA), 7% (w/v) sodium dodecyl sulphate, 6.7% (v/v) deionized formamide] at 65°C.

A human LH receptor probe (provided by Dr M.Atger) was labelled with 50 µCi [^{32}P]dCTP by the random priming method using a commercial kit (Amersham International plc) and added to the hybridization buffer. The membranes were hybridized overnight at 65°C. The following day the membranes were washed twice with 2× SSC and once with 2× SSC/0.1% SDS for 15 min at 65°C. They were laid onto a phosphor screen for 36 h and visualized using a phosphorimager computer (Molecular Dynamics, Maidstone, UK). To correct for minor differences in RNA loading, the blots were stripped in stripping buffer (5 mM Tris, pH 8.0, 0.3 mM EDTA, 0.1× Denhardt's reagent) for 2 h at 65°C. The blots were then probed for 18S RNA using an oligonucleotide probe as described previously (Brooks *et al.*, 1992). The molecular size of the transcripts was determined by running commercial RNA markers (Promega, Southampton, UK) in an adjacent lane.

In-situ hybridization

Isotopic in-situ hybridization was performed on frozen sections using ^{35}S-labelled riboprobes. Antisense and sense riboprobes incorporating ^{35}S-labelled UTP (Amersham International plc) were synthesized using a commercial kit (Promega). The antisense probe was generated from the plasmid vector linearized by *Hin*dIII (Promega) using T3 RNA polymerase (Promega). The sense probe was used as a negative control. This was generated from the plasmid vector linearized by *Eco*RI (Promega) using T7 RNA polymerase (Promega).

Frozen sections (5 µm) on poly-L-lysine (50 µg/l)-coated slides were thawed quickly and fixed in 4% paraformaldehyde for 5 min at room temperature. After washing in 0.1 M sodium phosphate, slides were rinsed firstly in water and then in 0.1 M triethanolamine (TEA), pH 8.0. The slides were then acetylated in 0.25% (v/v) acetic anhydride (BDH Laboratory Supplies, Poole, UK) in TEA. After acetylation, the slides were washed in 2× SSC and dehydrated through graded alcohols. The slides were then dried under a vacuum in a desiccator for 1 h at room temperature. In all, 100 µl of hybridization buffer [50% deionized formamide, 10% dextran sulphate, 1× Denhardt's solution, 0.5 mg/ml yeast tRNA, 10 mM dithiothreitol (DTT), 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0] containing 1×10^6 c.p.m. radiolabelled probe were added to each section. The slides were covered with a hydrophobic coverslip (Gel Bond; ICN Biomedical Ltd, High Wycombe, UK) and incubated overnight at 55°C in a moist chamber.

The following day the coverslips were washed off in 4× SSC. After several rinses in 4× SSC, the slides were treated with RNAse A (20 µg/ml) in RNAse buffer (10 mM Tris, 1 mM EDTA, 0.5 M NaCl, pH 8.0) for 30 min at 37°C. The sections were desalted by rinsing in 2× SSC/1 mM DTT, followed by 1× SSC/1 mM DTT and 0.5× SSC/1 mM DTT at room temperature. The slides were then washed for 30 min in 0.1× SSC at 70°C in a shaking water bath. After rinsing in 0.1× SSC/1 mM DTT at room temperature, the sections were dehydrated through graded alcohols containing 1 mM DTT and 0.08× SSC, washed in pure ethanol and allowed to dry. These slides were then dipped in Kodak NTB-2 photographic emulsion (IBI Ltd, Cambridge, UK) and incubated in the dark for 21 days. They were developed (Kodak D-19) and fixed (Kodak Unifix) at 15°C in the dark. After rinsing in running tap water, sections were counterstained with haematoxylin and mounted in Pertex mounting medium (Cellpath, Hemel Hempstead, UK).

In-situ ligand binding

In-situ ligand binding was performed using a modification of the method described by Molenaar *et al.* (1993). Frozen sections of 5 µm were cut onto poly-L-lysine-coated slides and stored at –70°C until use. They were thawed quickly and incubated in binding buffer [50 mM HEPES, 5 mM $MgCl_2$, 0.3% (w/v) BSA, pH 7.4] at room temperature for 20 min. Excess buffer was removed and 10 000 c.p.m. [^{125}I]LH (Chelsea Reagent; Hammersmith Hospital, London, UK) or 10 000 c.p.m. [^{125}I]LH with an excess (20 IU) of cold HCG (Profasi; Serono Laboratories), in binding buffer, were added to each slide for 2 h at room temperature. The slides were washed briefly four times in 0.05 M Tris, pH 7.4, at 4°C, dipped in distilled water and allowed to dry for 3 h at 4°C. They were then dipped in photographic emulsion (Kodak NTB-2) and stored at 4°C for 3 days in the dark. After developing (Kodak D-19) and fixing (Kodak Unifix) at 15°C in the dark, the slides were washed in water, counterstained with haematoxylin, dehydrated through graded alcohols and mounted in Pertex mounting medium.

Analysis of the sections

The distribution and number of silver grains were analysed by dark-field microscopy after image capture using computer-based image analysis systems. To quantify the results of the in-situ hybridization, the area proportion of silver grains over the steroidogenic cells was measured in five random fields for each section using an image analysis program (NIH Image 1.55; NIH, Bethesda, MD, USA). Acellular areas or areas without the steroidogenic cells were ignored. Only sections from the same run, performed under carefully controlled conditions, were analysed. The results of the in-situ ligand binding were analysed in a similar fashion except that the grain distribution in this case allowed the measurement of absolute numbers of grains. Grains were counted using the Cue-2 image analysis system (Olympus Optical Co. UK Ltd, London, UK). In each case the grain density

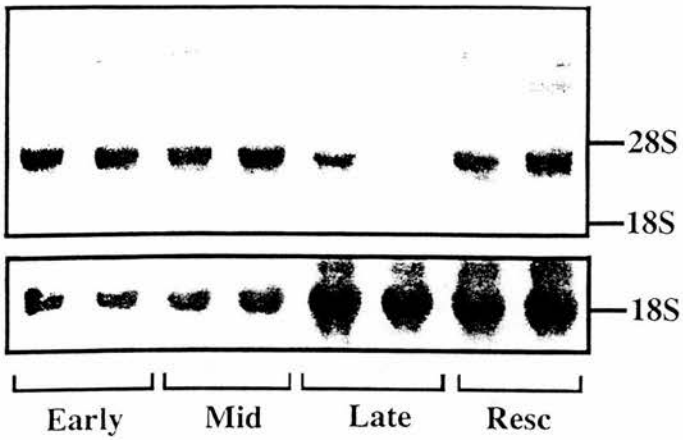

Figure 1. Northern blotting for luteinizing hormone (LH) receptor in human corpora lutea. The positions of the 28S and 18S ribosomal RNA bands are indicated on the right. The expression of 18S RNA is shown to control for differences in RNA loading. LH receptor mRNA could be detected in corpora lutea from all stages of the luteal phase [early (LH+1 to LH+5), mid- (LH+6 to LH+10), late (LH+11 to LH+14)] and after luteal rescue with exogenous human chorionic gonadotrophin (Resc).

was compared at each stage of the luteal phase using an analysis of variance with a 5% level of statistical significance.

Results

Plasma progesterone concentrations

The classification of the corpora lutea by serial urinary LH measurements agreed with the luteal phase dating of endometrial biopsies using the method of Li *et al.* (1988). As reported previously (Duncan *et al.*, 1996), the plasma progesterone concentrations were 36.4 ± 9.3 nmol/l in the early luteal samples, 40.4 ± 9.9 nmol/l in the mid-luteal samples and 18.8 ± 12.8 nmol/l in the late luteal samples. After luteal rescue by exogenous HCG the plasma progesterone concentrations had increased to 52.8 ± 1.1 nmol/l.

Detection of LH/HCG receptor mRNA

A major 4.5 kb band and minor 6.8–7.2 kb bands were detected by Northern blotting in the human corpus luteum (Figure 1). These are consistent with the size of the major LH receptor transcripts reported previously in the corpus luteum of primates (Ravindranath *et al.*, 1992; Nishimori *et al.*, 1995). LH/HCG receptor mRNA could be detected in corpora lutea from all stages of the luteal phase and after luteal rescue with HCG. The LH/HCG receptor mRNA was localized to the steroidogenic cells of the corpus luteum by in-situ hybridization (Figure 2a). No specific localization was seen in any of the control sections incubated with the sense probe (Figure 2b). Messenger RNA for the LH/HCG receptor could be detected, by in-situ hybridization at all stages of the functional luteal phase and after luteal 'rescue' with exogenous HCG (Figure 2c and d). No significant differences in the level of LH/HCG receptor expression, as measured by grain density, were found between different stages of the luteal phase (Figure 3). The expression of LH/HCG receptor mRNA during luteal rescue was similar to that seen in mid-luteal phase corpus luteum.

Detection of LH binding sites

Specific binding sites for LH were detected in the steroidogenic cells of the normal corpus luteum (Figure 4a and b). These binding sites were detected in all corpora lutea from each stage of the luteal phase. In addition, they could also be found after exposure to logarithmically increasing doses of HCG *in vivo* to simulate maternal recognition of pregnancy (Figure 4c). No specific binding of LH was observed in the negative control sections (Figure 4d). When the binding sites were quantified, using grain counting, no significant differences were observed at any stage of the luteal phase or after luteal rescue with HCG (Figure 5). The LH/HCG receptor protein, as measured by specific binding, was maintained during maternal recognition of pregnancy at similar levels to the mid-luteal phase.

Discussion

This paper describes the expression of the LH/HCG receptor in human corpora lutea throughout the functional luteal phase and after luteal 'rescue' with exogenous HCG. Messenger RNA for the LH/HCG receptor has been demonstrated previously in primate corpus luteum at different stages of the luteal phase (Ravindranath *et al.*, 1992; Nishimori *et al.*, 1995). Ravindranath *et al.* (1992) studied the expression of the LH receptor in corpora lutea of cynomolgus monkeys. They reported that LH receptor mRNA increased in the early luteal phase and was continually expressed in the corpus luteum throughout the luteal phase. Our data confirm that the LH/HCG receptor is expressed throughout the functional life-span of the primate corpus luteum.

These observations differ slightly from those of Nishimori *et al.* (1995), who reported a significant reduction in the expression of LH receptor mRNA in the late luteal phase. Although levels of LH receptor mRNA tended to be lower in the late luteal corpus luteum, this did not reach statistical significance in our study. This is unlikely to be explained by the number of corpora lutea examined because the same number were investigated in each study. We studied the expression of the LH receptor by quantifying the grain density over steroidogenic cells after in-situ hybridization. Nishimori *et al.* (1995) used Northern blotting of whole gland mRNA to quantify LH receptor expression, and the difference may reflect these different techniques. However, as the LH receptor is not expressed in the corpus luteum after menstruation (Ravindranath *et al.*, 1992; Nishimori *et al.*, 1995), it is clear that its expression is switched off at the completion of functional luteolysis. As the definition of the late luteal phase differs in each study, it is possible that the late luteal glands studied by Nishimori *et al.* (1995) were closer to the completion of functional luteolysis than in our study.

It has been suggested that the stability of transcribed LH receptor mRNA may be decreased to prevent translation into the receptor protein (Lu *et al.*, 1993). We used in-situ ligand

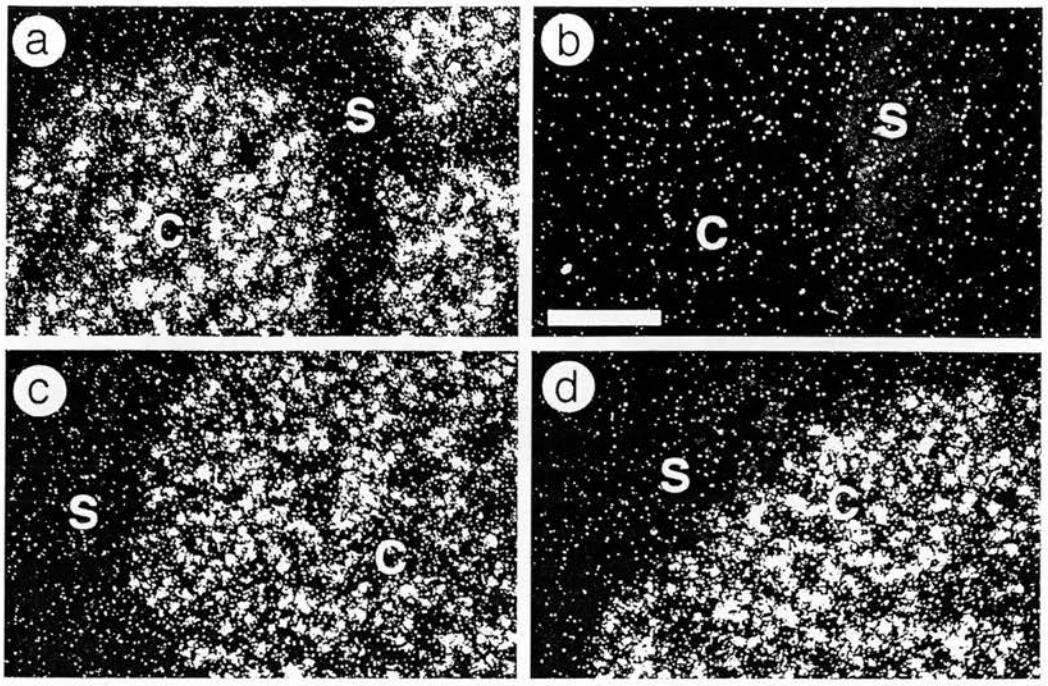

Figure 2. In-situ hybridization for luteinizing hormone (LH) receptor mRNA in the human corpus luteum. (**a**) Dark field of corpus luteum from the early luteal phase. Many more grains are seen over the steroidogenic cells (C) than the surrounding stroma (S). (**b**) Negative control dark-field serial section of (**a**) after in-situ hybridization with the sense riboprobe, showing no difference in background hybridization between the steroidogenic cells (C) and the surrounding stroma (S). (**c**) Dark-field late luteal corpus luteum showing LH receptor mRNA in the steroidogenic cells (C) but not in the stroma (S). (**d**) Dark field of LH receptor mRNA in a corpus luteum after rescue with exogenous human chorionic gonadotrophin. Expression is maintained in the steroidogenic cells (C) and is absent from the surrounding stroma (S). Scale bar = 200 μm.

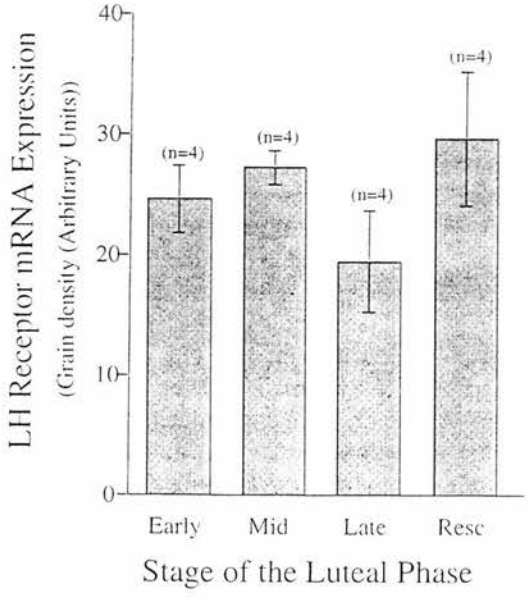

Figure 3. Luteinizing hormone (LH) receptor mRNA in the human corpus luteum as measured by grain density after in-situ hybridization. No differences were seen in the expression of LH receptor message in the early, mid- and late luteal phases or after luteal rescue with exogenous human chorionic gonadotrophin. Values are means ± SEM.

binding to identify the LH receptor protein in human corpus luteum. Numerous studies have demonstrated the presence of LH/HCG receptors in human corpus luteum using ligand binding assays (Rao *et al.*, 1977; McNeilly *et al.*, 1980; Shima *et al.*, 1987). Although these studies reported that LH receptor binding was reduced in the late luteal phase, it has subsequently become clear that when receptor occupancy was taken into account, levels of total receptor are similar throughout the luteal phase (Bramley *et al.*, 1987; Yeko *et al.*, 1989). We have confirmed that the LH receptor protein, in addition to mRNA, is maintained in the corpus luteum throughout its functional life-span.

The cause of functional luteolysis in the primate is not clear (Behrman *et al.*, 1993). The decline in progesterone secretion in the late luteal phase is not associated with falling serum LH concentrations (Hutchison *et al.*, 1986). This suggests that the corpus luteum is becoming increasingly insensitive to LH. Expression of the LH/HCG receptor is regulated both transcriptionally and post-transcriptionally (Segaloff and Ascoli, 1993). However, the continued presence of both the receptor mRNA and protein, as measured by ligand binding, suggests that the luteal LH/HCG receptor is maintained while progesterone production is falling. This is consistent with the findings of Cameron and Stouffer (1982), who compared cell membrane LH binding with progesterone production in macaque corpus luteum. These data suggest that functional luteolysis may be associated with an increasing block to steroidogenesis downstream of LH/HCG receptor binding.

We found that LH receptor mRNA and protein were maintained during maternal recognition of pregnancy with exogenous HCG. Previous studies have investigated the luteal LH/HCG receptor by binding assays early in human pregnancy (Rao *et al.*, 1977; McNeilly *et al.*, 1980; Bramley *et al.*, 1987;

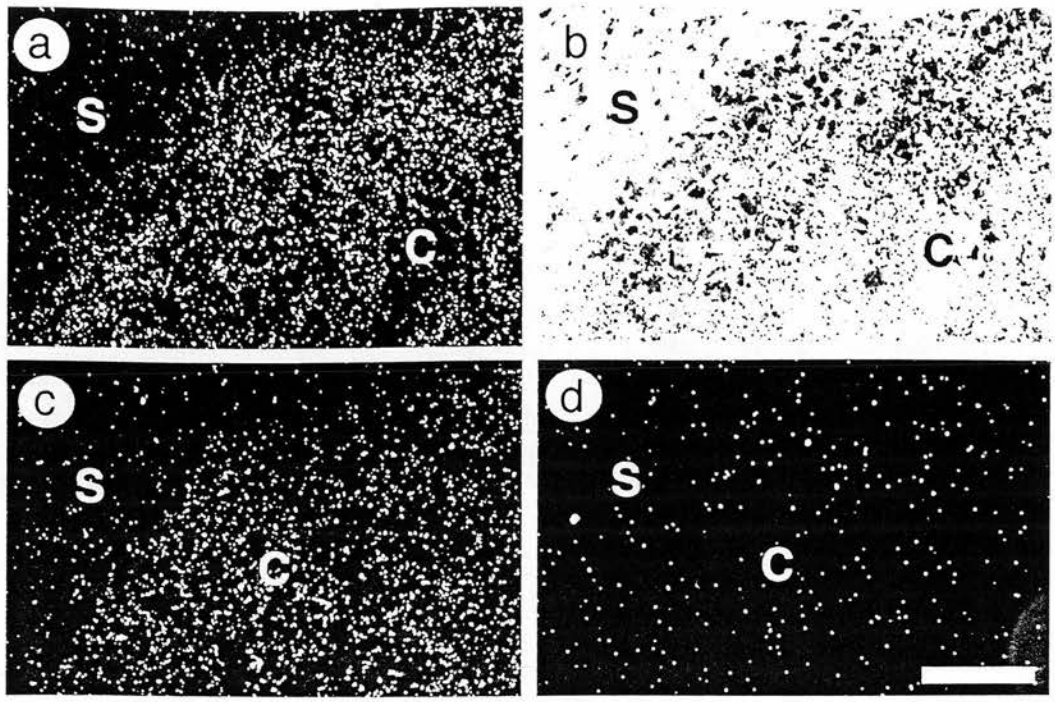

Figure 4. Demonstration of the luteinizing hormone (LH) receptor in the human corpus luteum by in-situ ligand binding. (**a**) Dark field of corpus luteum from the mid-luteal phase. Many more grains are seen over the steroidogenic cells (C) than the surrounding stroma (S). (**b**) Light field of section (**a**) showing the localization of the steroidogenic cells (C) and the surrounding stroma (S). (**c**) Dark field of the LH receptor in the corpus luteum after rescue with exogenous human chorionic gonadotrophin, showing continued LH binding in the steroidogenic cells (C) but not in the stroma (S). (**d**) Dark field of negative control serial section of (**c**) showing no specific binding to the steroidogenic cells (C) or the surrounding stroma (S). Scale bar = 100 μm.

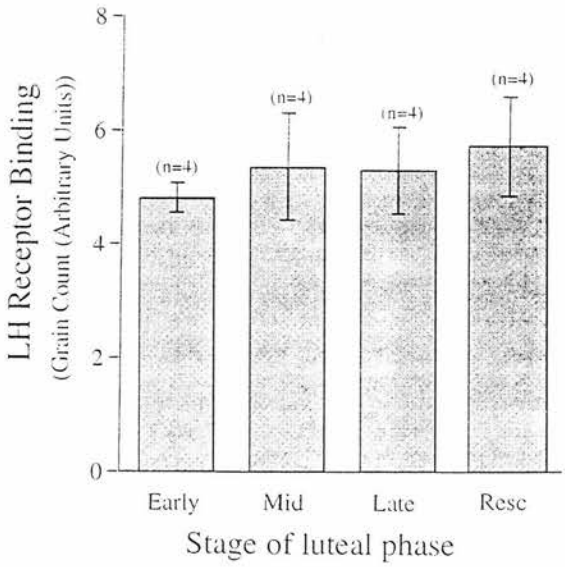

Figure 5. Luteinizing hormone (LH) receptors in the human corpus luteum as measured by grain counting after in-situ ligand binding. No differences were seen in the number of LH binding sites in the early, mid- and late luteal phases or after luteal rescue with exogenous human chorionic gonadotrophin. Values are means ± SEM.

Dawood and Khan-Dawood, 1994). Concentrations of LH receptor were variable but were much lower than in mid-luteal corpus luteum. In addition, LH/HCG receptor mRNA has now been identified in the corpus luteum of pregnant women (Nishimori *et al.*, 1995). Like receptor binding, levels of mRNA expression were much lower than in mid-cycle corpus luteum. However, in each case, material from the corpus luteum of ectopic pregnancies was investigated. In established pregnancies, maternal recognition of pregnancy has taken place, and although luteal progesterone production is continuing, it is beginning to decline (Tovanabutra *et al.*, 1993). In addition, ectopic pregnancies have suboptimal serum HCG and progesterone concentrations (Barnea *et al.*, 1986; Ledger *et al.*, 1994) and are usually associated with vaginal bleeding (Li *et al.*, 1991). It appears that luteal LH/HCG receptor expression is maintained to a greater degree during maternal recognition of uterine pregnancy than in established ectopic pregnancy.

These data are in agreement with those of Ottobre and Stouffer (1986), who studied LH binding to homogenates of rhesus monkey corpora lutea after exogenous HCG administration. They found that, although the number of available receptors dropped, the total number of receptors remained the same. However, it was not clear if these receptors were membrane bound or if recycling of receptors was occurring. Using in-situ hybridization in association with in-situ ligand binding, we have confirmed the continued presence of the LH/HCG receptor and demonstrated the continued transcription of receptor mRNA. These data provide strong evidence that the LH receptor is not down-regulated during maternal recognition of pregnancy in the primate.

In contrast, there is considerable evidence for ligand-induced down-regulation of the LH receptor in other cellular systems. In cell lines expressing the LH receptor, exposure to ligand causes a down-regulation of LH receptor binding (Segaloff and Ascoli, 1993). This loss of ligand binding activity is

associated with a loss of LH receptor mRNA (Hoffman *et al.*, 1991). In rat corpus luteum, LH receptor mRNA could not be detected 24 h after ligand-induced down-regulation (Peegel *et al.*, 1994). LH receptor mRNA expression in the same corpora lutea recovered, but not until 72 h after a single exposure to ligand. In addition, in adult rat testis, exposure to HCG resulted in a prolonged down-regulation of the LH receptor message (Pakarinen *et al.*, 1990). In ruminants, the administration of HCG was also associated with a marked down-regulation of luteal LH receptors (Niswender *et al.*, 1985). Although the LH receptor can be up-regulated in the growing follicle of the rat (LaPolt *et al.*, 1990), this has not been described in the corpus luteum. In the corpus luteum of non-primate species, it is clear that LH receptors are down-regulated both *in vitro* and *in vivo* by exposure to HCG.

Thus it seems likely that the effect of HCG on the LH receptor is species specific. Caldwell *et al.* (1980) treated luteal phase rats and women with equivalent doses of HCG. In the rat, both progesterone production and LH receptor content fell significantly, whereas in women, luteal progesterone production increased. The mechanisms of luteolysis and maternal recognition of pregnancy differ in primates and non-primate species (Auletta and Flint, 1988). It appears that, by using an LH-like chorionic gonadotrophin to maintain progesterone production from the corpus luteum, primates have adapted to overcome down-regulation of the LH/HCG receptor during maternal recognition of pregnancy.

The LH receptor is regulated by other mechanisms in addition to transcription and translation. Desensitization of the receptor to its ligand, with uncoupling from second messenger systems, has been reported *in vitro* and *in vivo* (Segaloff and Ascoli, 1993). Such desensitization may explain why increasing doses of HCG are required to maintain progesterone production during pregnancy. In addition, there are multiple transcripts of the LH receptor regulated by alternative splicing (Themmen *et al.*, 1994). Bacich *et al.* (1994), using an ovine model, showed that full-length receptor mRNA was a minority of the LH receptor mRNA species detected in the corpus luteum. Most mRNA species coded for truncated or non-functioning receptors. This alternative splicing of the LH receptor has also been reported in other species (Aatsinki *et al.*, 1992; VuHai-LuuThi *et al.*, 1992). However, the functional significance of these transcripts is not yet clear and it is not known whether they are expressed in corpora lutea of primates.

In summary, this study shows that both LH receptor mRNA and protein are maintained in human corpus luteum during maternal recognition of pregnancy. The lack of down-regulation is further evidence that primates and non-primates exhibit different mechanisms to control the function of the corpus luteum.

Acknowledgements

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References

Aatsinki, J.T., Pietilä, E.M., Lakkakorpi, J.T. and Rajaniemi, H.J. (199 Expression of the LH/CG receptor gene in rat ovarian tissue is regulate by an extensive alternative splicing of the primary transcript. *Mol. Cel Endocrinol.*, **84**, 127–135.

Auletta, F.J. and Flint, A.P.F. (1988) Mechanisms controlling corpus luteu function in sheep, cows, nonhuman primates, and women especially i relation to the time of luteolysis. *Endocr. Rev.*, **9**, 88–105.

Bacich, D.J., Rohan, R.M., Norman, R.J. and Rodgers, R.J. (199 Characterization and relative abundance of alternatively spliced luteinizin hormone receptor messenger ribonucleic acid in the ovine ovar *Endocrinology*, **135**, 735–744.

Barnea, E.R., Oelsner, G., Benveniste, R. *et al.* (1986) Progesterone, estradio and β-human chorionic gonadotropin secretion in patients with ectopi pregnancy. *J. Clin. Endocrinol. Metab.*, **62**, 529–531.

Behrman, H.R., Endo, T., Aten, R.F. and Musicki, B. (1993) Corpus luteu function and regression. *Reprod. Med. Rev.*, **2**, 153–180.

Bramley, T.A., Stirling, D., Swanston, I.A. *et al.* (1987) Specific binding site for gonadotrophin-releasing hormone, LH/chorionic gonadotrophin, low density lipoprotein, prolactin and FSH in homogenates of human corpu luteum. II: Concentrations throughout the luteal phase of the menstru cycle and early pregnancy. *J. Endocrinol.*, **113**, 317–327.

Brooks, J., Crow, W.J., McNeilly, J.R. and McNeilly, A.S. (1992) Relationshi between gonadotropin subunit gene-expression, gonadotropin-releasin hormone receptor content and pituitary and plasma gonadotropi concentrations during the rebound release of FSH after treatment of ewe with bovine follicular-fluid during the luteal phase of the cycle. *J. Mo Endocrinol.*, **8**, 109–118.

Caldwell, B.V., Rotchell, Y.E., Pang, C.Y. *et al.* (1980) Comparative study o high-dose chorionic gonadotropin on the human and rat corpus luteum an effect of gonadotropin-releasing hormone on human luteal function. *Am. J Obstet. Gynecol.*, **136**, 458–464.

Cameron, J.L. and Stouffer, R.L. (1982) Gonadotropin receptors of the primat corpus luteum. II. Changes in available luteinizing hormone- and chorioni gonadotropin-binding sites in macaque corpus luteal membranes during th nonfertile menstrual cycle. *Endocrinology*, **110**, 2068–2073.

Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation b acid guanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Cole, F.E., Weed, J.C., Schneider, G.T. *et al.* (1973) The gonadotropin recepto of the human corpus luteum. *Am. J. Obstet. Gynecol.*, **117**, 87–95.

Dawood, M.Y. and Khan-Dawood, F.S. (1994) Human corpus luteum: huma chorionic gonadotropin receptors during ectopic pregnancy. *Fertil. Steril.* **62**, 711–715.

Djahanbakhch, O., McNeilly, A.S., Hobson, B.M. and Templeton, A.A (1981a) A rapid luteinizing hormone radioimmunoassay for the predictio of ovulation. *Br. J. Obstet. Gynaecol.*, **88**, 1016–1020.

Djahanbakhch, O., Swanston, I.A., Corrie, J.E.T. and McNeilly, A.S. (1981b Prediction of ovulation by progesterone. *Lancet*, **ii**, 1164–1165.

Duncan, W.C., McNeilly, A.S. and Illingworth, P.J. (1996) Expression o tissue inhibitor of metalloproteinases-1 in the human corpus luteum afte luteal rescue. *J. Endocrinol.*, **148**, 59–67.

Fraser, H.M., Abbott, M., Laird, N.C. *et al.* (1986) Effects of an LHRH antagonist on the secretion of LH, FSH, prolactin and ovarian steroids a different stages of the luteal phase in the stumptailed (*Macca arctoides*) *J. Endocrinol.*, **111**, 83–89.

Hoffman, Y.M., Peegel, H., Sprock, M.J.E. *et al.* (1991) Evidence that huma chorionic gonadotropin/luteinizing hormone receptor down regulatio involves decreased levels of receptor message ribonucleic acid *Endocrinology*, **128**, 338–393.

Hutchison, J.S. and Zeleznik, A.J. (1984) The rhesus monkey corpus luteun is dependent on pituitary gonadotropin secretion throughout the luteal phas of the menstrual cycle. *Endocrinology*, **115**, 1780–1786.

Hutchison, J.S., Nelson, P.B. and Zeleznik, A.J. (1986) Effects of differen gonadotropin pulse frequencies on corpus luteum function during th menstrual cycle of rhesus monkeys. *Endocrinology*, **119**, 1964–1971.

Illingworth, P.J., Reddi, K., Smith, K. and Baird, D.T. (1990) Pharmacologic 'rescue' of the corpus luteum results in increased inhibin production. *Clin Endocrinol.*, **33**, 323–332.

LaPolt, P., Oikawa, M., Jia, X.-C. *et al.* (1990) Gonadotropin-induced up- and down-regulation of rat ovarian LH receptor message levels during follicular growth, ovulation and luteinization. *Endocrinology*, **126**, 3277–3279.

Ledger, W.L., Sweeting, V.M. and Chaterjee, S. (1994) Rapid diagnosis of early ectopic pregnancy in an emergency gynaecology service — are measurements of progesterone and intact and free β human chorionic gonadotrophin helpful? *Hum. Reprod.*, **9**, 157–160.

Lenton, E.A. and Woodward, A.J. (1988) The endocrinology of conception cycles and implantation in women. *J. Reprod. Fertil. Suppl.*, **36**, 1–15.

[illegible], T.C., Rogers, A.W., Dockery, P. *et al.* (1988) A new method of histologic dating of human endometrium in the luteal phase. *Fertil. Steril.*, **50**, 52–60.

[illegible], T.C., Tristram, A., Hill, A.S. and Cooke, I.D. (1991) A review of 254 ectopic pregnancies in a teaching hospital in the Trent Region, 1977–1990. *Hum. Reprod.*, **6**, 1002–1007.

Lu, D.L., Peegel, H., Mosier, S.M. and Menon, K.M.J. (1993) Loss of lutropin/human choriogonadotropin receptor messenger ribonucleic acid during ligand-induced downregulation occurs post transcriptionally. *Endocrinology*, **132**, 235–240.

McNeilly, A.S., Kerin, J., Swanston, I.A. *et al.* (1980) Changes in the binding of human chorionic gonadotrophin/luteinizing hormone, follicle-stimulating hormone and prolactin to human corpora lutea during the menstrual cycle and pregnancy. *J. Endocrinol.*, **87**, 315–325.

Minegish, T., Nakamura, K., Takakura, Y. *et al.* (1990) Cloning and sequencing of human LH/hCG receptor cDNA. *Biochem. Biophys. Res. Commun.*, **172**, 1049–1054.

Molenaar, P., O'Reilly, G., Sharkey, A. *et al.* (1993) Characterization and localization of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium. *Circ. Res.*, **72**, 526–538.

Nishimori, K., Dunkel, L., Hsueh, A.J.W. *et al.* (1995) Expression of luteinizing hormone and chorionic gonadotropin receptor messenger ribonucleic acid in human corpora lutea during menstrual cycle and pregnancy. *J. Clin. Endocrinol. Metab.*, **80**, 1444–1448.

Niswender, G.D., Schwall, R.H., Fitz, T.A. *et al.* (1985) Regulation of luteal function in domestic ruminants: new concepts. *Rec. Prog. Hormone Res.*, **41**, 101–151.

Ottobre, J.S. and Stouffer, R.L. (1986) Receptors for chorionic gonadotropin in the corpus luteum of the rhesus monkey during simulated early pregnancy: lack of down-regulation. *Endocrinology*, **119**, 1594–1602.

Pakarinen, P., Vihko, K.K., Voutilainen, R. and Huhtaniemi, I. (1990) Differential response of luteinizing hormone receptor and steroidogenic enzyme gene expression to human chorionic gonadotropin stimulation in the neonatal and adult rat testis. *Endocrinology*, **127**, 2469–2474.

Peegel, H., Randolph, J., Jr, Midgely, A.R. and Menon, K.M.J. (1994) *In situ* hybridization of luteinizing hormone/human chorionic gonadotropin receptor messenger ribonucleic acid during hormone-induced down-regulation and the subsequent recovery in rat corpus luteum. *Endocrinology*, **135**, 1044–1051.

Rao, Ch.V., Griffin, L.P. and Carman, F.R., Jr (1977) Gonadotropin receptors in human corpora lutea of the menstrual cycle and pregnancy. *Am. J. Obstet. Gynecol.*, **128**, 146–153.

Ravindranath, N., Little-Ihrig, L.L. and Zeleznik, A.J. (1992) Characterization of the levels of messenger ribonucleic acid that encode for luteinizing hormone receptor during the luteal phase of the primate menstrual cycle. *J. Clin. Endocrinol. Metab.*, **74**, 779–785.

Segaloff, D.L. and Ascoli, M. (1993) The lutropin/choriogonadotropin receptor. 4 years later. *Endocr. Rev.*, **14**, 324–347.

Shima, K., Kitayama, S. and Nakano, R. (1987) Gonadotropin binding sites in human ovarian follicles and corpora lutea during the menstrual cycle. *Obstet. Gynecol.*, **69**, 800–806.

Themmen, A.P.N., Kraaij, R. and Grootegoed, J.A. (1994) Regulation of gonadotropin receptor gene expression. *Mol. Cell. Endocrinol.*, **100**, 15–19.

Tovanabutra, S., Illingworth, P.J., Ledger, W.L. *et al.* (1993) The relationship between peripheral immunoactive inhibin, human chorionic gonadotrophin, oestradiol and progesterone during human pregnancy. *Clin. Endocrinol.*, **38**, 101–107.

VuHai-LuuThi, M.T., Misrahi, M., Houllier, A. *et al.* (1992) Variant forms of the pig lutropin/choriogonadotropin receptor. *Biochemistry*, **31**, 8377–8383.

Yamoto, M., Nishimori, K. and Nakana, R. (1988) Masked gonadotropin-binding sites in human corpora lutea during menstrual cycle and pregnancy. *Fertil. Steril.*, **50**, 239–244.

Yeko, T.R., Khan-Dawood, F.S. and Dawood, M.Y. (1989) Human corpus luteum: luteinizing hormone and chorionic gonadotropin receptors during the menstrual cycle. *J. Clin. Endocrinol. Metab.*, **68**, 529–534.

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Induced luteolysis in the primate: rapid loss of luteinizing hormone receptors

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The molecular mechanisms involved in luteolysis are still unclear in the primate. This study aimed to investigate the effect of induced luteolysis on the ovarian luteinizing hormone (LH) receptor and the steroidogenic enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD) in the marmoset monkey. Luteolysis was induced in the mid-luteal phase either directly by systemic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), or indirectly by LH withdrawal using systemic gonadotrophin releasing hormone antagonist ($GnRH_{ant}$) treatment. The LH receptor was studied by isotopic mRNA in-situ hybridization and in-situ ligand binding and 3β-HSD expression was studied using isotopic mRNA in-situ hybridization and immunohistochemistry. Induced luteolysis was associated with a reduction in the expression of LH receptor ($P < 0.0001$) and 3β-HSD mRNA, closely followed by a reduction in the LH receptor ($P < 0.05$) and 3β-HSD protein concentrations within 24 h. There were no differences in the findings whether luteolysis was induced with $PGF_{2\alpha}$ or $GnRH_{ant}$. This study shows that disparate mechanisms to induce luteolysis in the primate result in an identical rapid loss of the LH receptor and 3β-HSD. In conclusion, induced luteolysis leads to rapid loss of the steroidogenic pathway in luteal cells.

Key words: corpus luteum/3β-hydroxysteroid dehydrogenase/immunohistochemistry/in-situ hybridization/luteinizing hormone receptor

Introduction

The molecular mechanisms of luteolysis in the primate and of how the functional and structural integrity of the corpus luteum are lost, are still unclear (Auletta and Flint, 1988; Behrman *et al.*, 1993). It has recently been shown that cell death by apoptosis (Juengel *et al.*, 1993; Fraser *et al.*, 1995a; Young *et al.*, 1997) and remodelling of the extracellular matrix by matrix metalloproteinase enzymes (Endo *et al.*, 1993), are likely to contribute to the loss of the structural integrity of the corpus luteum during luteolysis. The molecular mechanisms responsible for the loss of its functional integrity and its falling progesterone output, however, are still not fully understood.

The primate corpus luteum is dependent on the trophic support of luteinizing hormone (LH) from the pituitary gland. LH binds specifically to the LH receptor, a seven transmembrane region G-protein-coupled receptor (Segaloff and Ascoli, 1993) on the surface of luteal cells, to stimulate steroidogenic enzymes to produce progesterone. Withdrawal of LH results in luteolysis (Fraser *et al.*, 1986), whereas human chorionic gonadotrophin (HCG), from the implanting blastocyst, acts through the LH receptor to maintain steroidogenesis and 'rescue' the corpus luteum. We have previously shown that during luteal 'rescue', the LH receptor is maintained and is not down-regulated by its ligand (Duncan *et al.*, 1996a).

There is a significant reduction in luteal LH receptors preceded by reduced receptor mRNA concentrations after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced luteolysis in ruminants (Guy *et al.*, 1995; Smith *et al.*, 1996). This is thought to contribute to the functional decline of the ruminant corpus luteum. However, in the monkey, LH receptor mRNA increased during the late-luteal phase (Ravindranath *et al.*, 1992a) when progesterone output declined. Similarly, in the human, LH receptor mRNA and binding activity can be detected in the late-luteal corpus luteum (Nishimori *et al.*, 1995; Duncan *et al.*, 1996a), but not in the corpus luteum after menstruation (Ravindranath *et al.*, 1992a; Nishimori *et al.*, 1995). In the primate, functional luteolysis is thought to occur initially in the presence of normal concentrations of LH receptors.

The primate corpus luteum is capable of recovering from a transient withdrawal of gonadotrophic support (Hutchison and Zeleznik, 1985) but not from natural functional luteolysis (Stouffer *et al.*, 1977). This study investigated the effect of co-ordinated induced luteolysis on LH receptor and 3β-hydroxysteroid dehydrogenase (3β-HSD) expression in the primate. The marmoset monkey was used, as luteolysis can be induced by systemic $PGF_{2\alpha}$, although uterine $PGF_{2\alpha}$ is not the natural luteolysin in this species. In addition, luteolysis can be induced by LH withdrawal using gonadotrophin releasing hormone antagonist ($GnRH_{ant}$). We aimed to discover whether $PGF_{2\alpha}$ had the same effect on luteal LH receptors in the primate as the ruminant, and whether induced luteolysis by withdrawal of gonadotrophic support had the same effect.

Materials and methods

Collection of tissue

Captive-bred common marmoset monkeys (*Callithrix jacchus jacchus*) were maintained in a colony which has been closed since 1973. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986. To confirm normal ovulatory cycles, plasma samples were collected by femoral venepuncture on alternate days

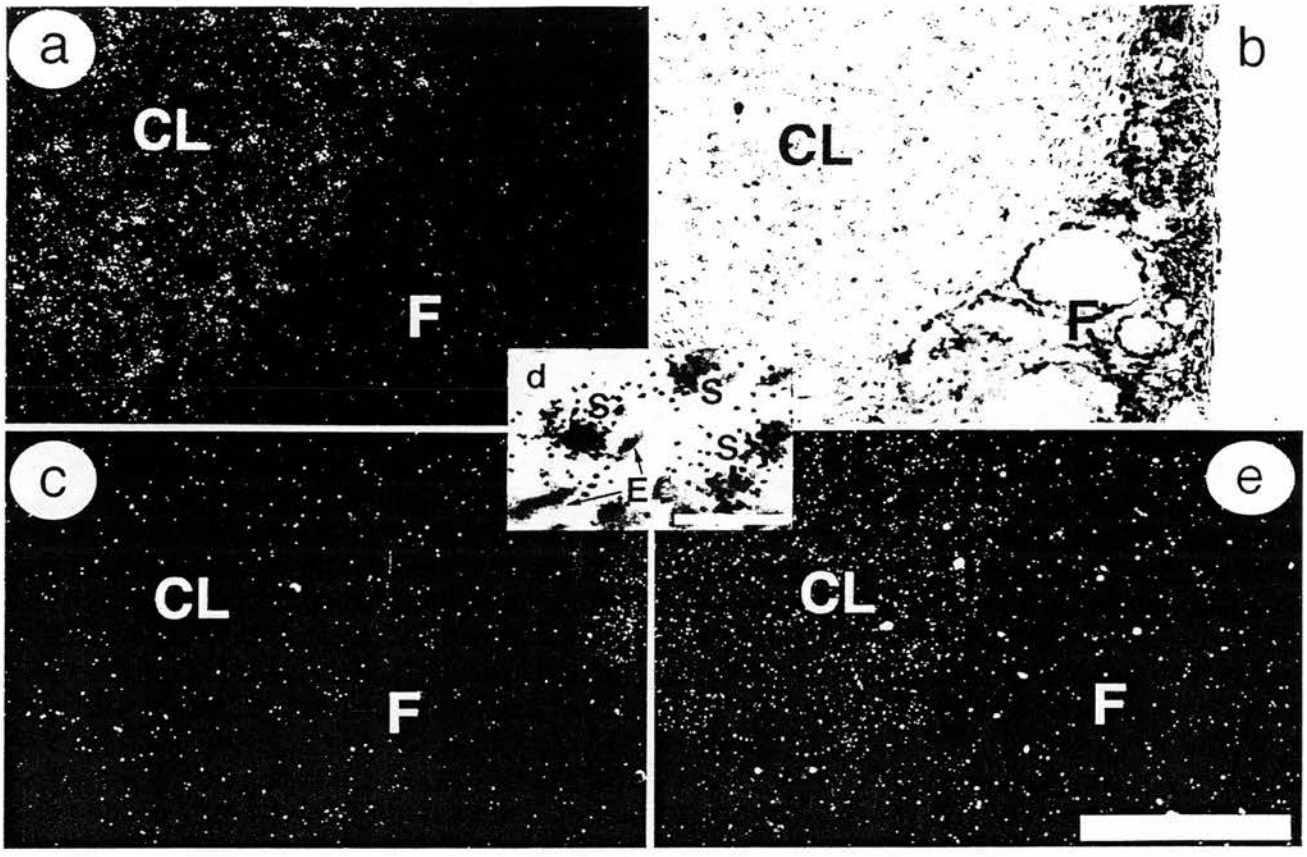

Figure 1. Luteinizing hormone (LH) receptor in the mid-luteal marmoset corpus luteum: (**a**) dark-field in-situ hybridization of LH receptor mRNA in the control mid-luteal corpus luteum (CL), no signal is seen in the stroma and tiny follicles (F); (**b**) light-field section of (**a**), showing the position of the corpus luteum (CL), stroma and tiny follicles (F); (**c**) dark-field serial negative control section of (**a**) using the sense probe for LH receptor mRNA, no signal can be seen in the corpus luteum (CL) or the tiny follicles (F); (**d**) light field higher power (scale bar = 25 μm) section of corpus luteum showing silver grains in steroidogenic cells (S) but not in endothelial-like cells (E); (**e**) dark-field serial section of (**a**) after LH binding studies showing binding over the corpus luteum (CL) and not over the stroma and tiny follicles (F). Scale bar = 200 μm.

and stored at –20°C until required. These samples were assayed for progesterone to determine the date of ovulation and the luteal phase duration (Smith *et al.*, 1990). The marmoset normally ovulates two to three follicles and has a functional luteal phase of 18–21 days.

Ovaries were collected on day 10 of the luteal phase as previously described (Fraser *et al.*, 1995a; Duncan *et al.*, 1996b). Ovaries were collected from untreated control animals (n = 4) and animals treated with: $PGF_{2\alpha}$ analogue, cloprostenol (1 μg i.m. injection; Planate, Coopers Animal Health Ltd, Crewe, Cheshire, UK), 24 h (n = 4) previously; $GnRH_{ant}$, antarelix ([*N*-Ac-D-Nal^1, D-pCl-Phe^2, D-Pal^3, D-(Hic)^6, Lys(iPr)^8, D-Ala^{10}]GnRH (Deghenghi *et al.*, 1993), 500 μg/kg s.c. injection, Europeptides (GEIE), Argenteuil, Val-D'Oise, France), 12 h (n = 2) and 24 h (n = 4) previously. Whole ovaries were snap-frozen in embedding medium (Tissue-Tek OCT compound; Miles Inc., Elkhart, IN, USA) and stored at –70°C until sectioning. Frozen sections (5 μm) were prepared from these ovaries and stored at –70°C until use. In addition, ovaries were also available from control (n = 4) and treated animals (n = 6) which had been fixed in 4% paraformaldehyde for 24 h and embedded in paraffin wax (Fraser *et al.*, 1995a). Five-micrometer sections were cut onto poly-L-lysine (50 μg/l)-coated slides for immunohistochemical analysis.

Source of reagents

All reagents used were obtained from Sigma Chemical, Poole, Dorset, UK unless otherwise indicated. A 1.5 kb cDNA construct, corresponding to nucleotide 542 to the last nucleotide of the open reading frame (2124), of the human LH receptor in pBluescript (Stratagene, Cambridge, Cambs, UK) was kindly supplied by Dr M. Atger of the Faculté de Médecine de Bicêtre, Université Paris-Sud, Le Kremlin-Bicêtre, France. Iodinated LH (Chelsea Reagent) was obtained commercially from Department of Chemical Pathology, Hammersmith Hospital, London, UK. The specific activity of the iodinated LH is 100 μCi/μg and 10 000 c.p.m. is equivalent to 45 pg. A 1.2 kb cDNA construct of human 3β-HSD in pIBI25 (Prof. J. I. Mason, Dept. of Chemical Biochemistry, University of Edinburgh, Edinburgh, UK) and a polyclonal antibody to human type I 3β-HSD was kindly supplied by Professor Van Luu-The, The CHUL Research Centre, Quebec, Canada. All restriction enzymes and RNA polymerases were obtained from Promega, Southampton, Hants, UK.

In-situ hybridization

Isotopic in-situ hybridization was performed on frozen sections using S^{35}-labelled riboprobes as described previously (Duncan *et al.*, 1996a). Antisense and sense LH receptor riboprobes incorporating S^{35}-labelled UTP (Amersham International plc, Aylesbury, Bucks, UK) were synthesized using a commercial kit (Promega). The antisense probe was generated from the plasmid vector linearized by HindIII using T3 RNA polymerase. The sense probe was used as a negative control. This was generated from the plasmid vector linearized by ECoR1 using T7 RNA polymerase. The antisense riboprobe for 3β-HSD

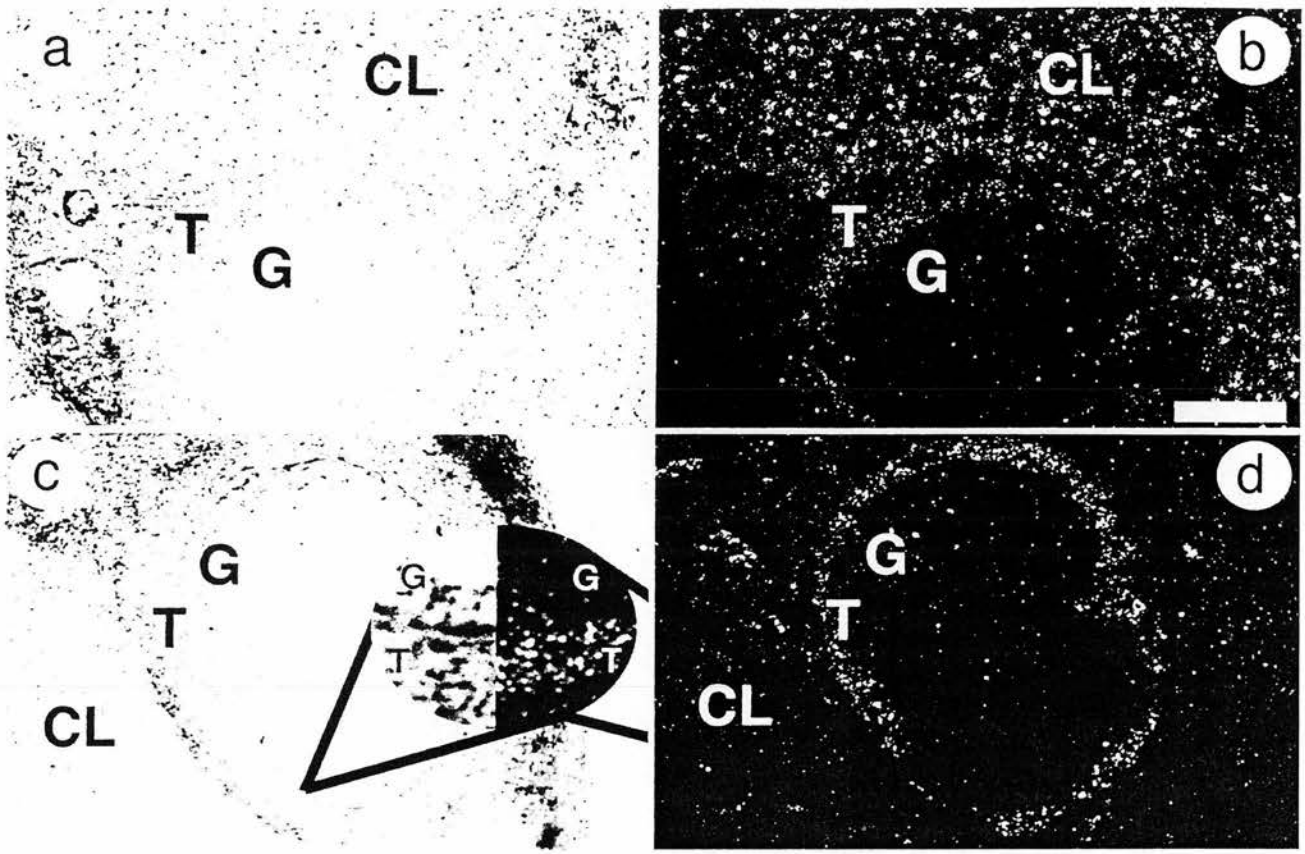

Figure 2. Luteinizing hormone (LH) receptor mRNA in the marmoset ovary after induced luteolysis: (**a**) light-field section of a mid-luteal marmoset ovary showing the corpus luteum (CL) and the theca (T) and granulosa cells of an antral follicle; (**b**) dark-field section of (**a**) after in-situ hybridization for LH receptor mRNA showing specific signal in the corpus luteum (CL) and theca (T) cells of the follicle, but not the granuolsa cells (G) of the follicle; (**c**) light-field section of a marmoset ovary 24 h after induced luteolysis with $GnRH_{ant}$ showing the corpus luteum (CL) and the theca (T) and granulosa (G) cells of an antral follicle; (**d**) dark-field section of (**c**) after in-situ hybridization for LH receptor mRNA showing no signal in the corpus luteum (CL) and granulosa cells (G) but maintenance of the signal in the theca cells (T) of the antral follicle. Scale bar = 200 μm. Insert shows higher power view of the follicle showing the grains to be localized to the theca (T) rather than granulosa (G) cells.

mRNA was generated by T7 RNA polymerase after plasmid linearization by SstI.

Frozen sections (5 μm) on poly-L-lysine (50 μg/l)-coated slides were quickly thawed and fixed in 4% paraformaldehyde for 5 min at room temperature. After washing in 0.1 M sodium phosphate, slides were rinsed firstly in water and then in 0.1 M triethanolamine (TEA) pH 8. The slides were then acetylated in 0.25% (v/v) acetic anhydride (BDH Laboratory Supplies, Poole, Dorset, UK) in TEA. After acetylation, the slides were washed in 2×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate) pH 7, and dehydrated through graded alcohols. The slides were then dried under vacuum in a desiccator for 1 h at room temperature. An aliquot of 100 μl of hybridization buffer (50% deionized formamide, 10% dextran sulphate, 1×Denhardt's solution, 0.5 mg/ml yeast tRNA, 10 mM dithiothreitol (DTT), 0.3 M NaCl, 10 mM Tris, 1 mM EDTA pH 8) containing 1×10^6 c.p.m. radiolabelled probe was added to each section. The slides were covered with a hydrophobic coverslip (Gel Bond; ICN Biomedical Ltd, High Wycombe, Bucks, UK) and incubated overnight at 55°C in a moist chamber.

The following day the coverslips were washed off in 4×SSC. After several rinses in 4×SSC, the slides were treated with RNase A (20 μg/ml) in RNase buffer (10 mM Tris, 1 mM EDTA, 0.5 M NaCl, pH 8) for 30 min at 37°C. The sections were de-salted by rinsing in 2×SSC/1 mM DTT, followed by 1×SSC/1 mM DTT and 0.5×SSC/1 mM DTT at room temperature. The slides were then washed for 30 min in 0.1×SSC at 70°C in a shaking water bath. After rinsing in 0.1×SSC/1 mM DTT at room temperature, the sections were dehydrated through graded alcohols containing 1 mM DTT and 0.08×SSC, washed in pure ethanol and allowed to dry. These slides were then dipped in photographic emulsion (Kodak NTB-2; IBI Ltd, Cambridge, Cambs, UK) and stored at 4°C for 18 days in the dark. After developing (Kodak D-19) and fixing (Kodak Unifix) at 15°C in the dark, the slides were washed in water, counterstained in haematoxylin, dehydrated through graded alcohols and mounted in Pertex mounting medium (Cellpath, Hemel Hempstead, Herts, UK).

In-situ ligand binding

In-situ ligand binding was performed as described previously (Duncan *et al.*, 1996a). Frozen sections (5 μm) were quickly thawed and incubated in binding buffer [50 mM HEPES, 5 mM $MgCl_2$, 0.3% (w/v) BSA, pH 7.4] at room temperature for 20 min. Excess buffer was removed and 10 000 c.p.m. of iodinated LH or 10 000 c.p.m. iodinated LH with excess (20 IU) of cold HCG (Profasi; Serono Laboratories, Welwyn Garden City, Herts, UK), in binding buffer was added to each slide for 2 h at room temperature. The slides were briefly washed four times in 0.05 M Tris pH 7.4 at 4°C, dipped in distilled water and allowed to dry for 3 h at 4°C. They were then

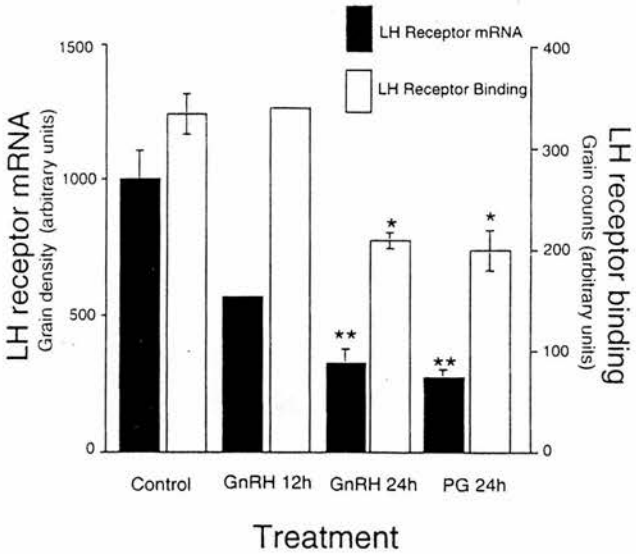

Figure 3. Luteinizing hormone (LH) receptor in marmoset corpora lutea after induced luteolysis. Grain density of LH receptor mRNA and grain counts of LH receptor binding in marmoset corpora lutea in the mid-luteal phase (control) (n = 4), 12 h after induced luteolysis with $GnRH_{ant}$ (GnRH 12 h) (n = 2), 24 h after induced luteolysis with $GnRH_{ant}$ (GnRH 24 h) (n = 4) and 24 h after induced luteolysis with $PGF_{2\alpha}$ (PG 24) (n = 4). Values are mean ± SEM: $^*P < 0.05$, $^{**}P < 0.0001$ (ANOVA).

dipped in photographic emulsion (Kodak NTB-2) and stored at 4°C for 3 days in the dark. After developing (Kodak D-19) and fixing (Kodak Unifix) at 15°C in the dark, the slides were washed in water, counterstained in haematoxylin, dehydrated through graded alcohols and mounted in Pertex mounting medium.

Immunohistochemistry

Sections were dewaxed in xylene, then rehydrated through graded alcohols to water. Sections then underwent microwave antigen retrieval at full power in four 5 min cycles in 0.01 M sodium citrate, pH 6.0. After standing for 20 min at room temperature, the slides were washed in 0.05 M Tris buffered saline, pH 8.0 (TBS) for 10 min. The sections were then incubated overnight at 4°C with the polyclonal rabbit anti-human 3β-HSD antiserum diluted 1:300 in TBS. On the following day, the sections were washed with TBS and then incubated with biotinylated goat anti-rabbit immunoglobulins (Dako Ltd, High Wycombe, Bucks, UK), diluted 1:500 in 20% normal goat serum (NGS) (SAPU, Carluke, Lanarkshire, UK), 5% BSA in TBS, for 1 h at room temperature. After being washed with TBS, sections were incubated with avidin–biotin alkaline phosphatase complex (Dako Ltd) for 1 h at room temperature, then washed again with TBS and developed with chromagen to give a red end product (Alkaline Phosphatase Substrate Kit I; Vector Laboratories, Peterborough, Cambs, UK). Sections were counterstained with haematoxylin, dehydrated through graded alcohols and cleared in xylene prior to mounting. Polyclonal rabbit IgG (Dako Ltd) at the same antibody concentration was used in place of the primary antibody, in serial sections, as a negative control.

Analysis of sections

The distribution and number of silver grains was analysed by dark-field microscopy after image capture, using computer-based image analysis systems. To quantify the results of the in-situ hybridization, the area proportion of silver grains over the steroidogenic cells was measured in five random fields for each section using an image analysis program (NIH Image 1.5; NIH Bethseda, MD, USA). Acellular areas or areas without the steroidogenic cells were ignored. Only sections from the same run, performed under carefully controlled conditions, were analysed. The results of the in-situ ligand binding were analysed in a similar fashion except that the grain distribution in this case allowed measurement of absolute numbers of grains. In each case, the grain density was compared in each treatment group using analysis of variance (ANOVA) with a 5% level of significance using a commercial statistics computer program (StatView 4.0; Abacus Concepts Inc., Berkeley, CA, USA).

Results

Progesterone concentrations

As described previously (Duncan *et al.*, 1996b), progesterone concentrations in the control animals were 330 ± 69 nmol/l (mean ± SEM). Functional luteal regression was observed in all animals treated with either the GnRH antagonist or the prostaglandin analogue (Fraser *et al.*, 1995b). Prostaglandin treatment resulted in a decline in progesterone concentrations to 22 ± 6 nmol/l after 24 h, and treatment with GnRH antagonist resulted in progesterone concentrations of 13 nmol/l after 12 h and 23 ± 11 nmol/l after 24 h. All progesterone concentrations after induced luteolysis were within the normal range of follicular phase concentrations in the marmoset (Smith *et al.*, 1990).

LH receptor after induced luteolysis

Messenger RNA for the LH receptor was detected by in-situ hybridization in corpora lutea of marmoset ovaries from the mid-luteal phase (Figure 1a,b). No specific signal was present in the negative control sections incubated with the sense riboprobe (Figure 1c). LH receptors were localized to individual steroidogenic cells within corpora lutea. No hybridization signal could be seen in cells without the morphological appearance of steroidogenic cells which expressed 3β-HSD, including endothelial cells (Figure 1d). The localization of LH receptor binding corresponded to the localization of LH receptor mRNA (Figure 1e). No specific binding was seen in negative control sections where excess cold HCG was added.

Messenger RNA for the LH receptor was also expressed in the theca cell layer of antral follicles in ovarian tissue from the mid-luteal phase (Figure 2a,b). After induced luteolysis, by $PGF_{2\alpha}$ or $GnRH_{ant}$ administration, LH receptor mRNA disappeared from corpora lutea but was maintained in the theca cell layer of antral follicles (Figure 2c,d). Luteal LH receptor mRNA concentrations fell after treatment to very low concentrations 12 and 24 h ($P < 0.0001$) after induced luteolysis (Figure 3). Luteal LH receptor binding was similar to controls 12 h after induced luteolysis but was significantly reduced by 24 h ($P < 0.05$) (Figure 3). Although the small numbers at 12 h precluded accurate statistical analysis, the loss of LH binding appeared to lag behind the loss of LH receptor mRNA (Figures 3, 4a–f). There were no differences in luteal LH receptor mRNA or binding where luteolysis was induced with $PGF_{2\alpha}$ or $GnRH_{ant}$.

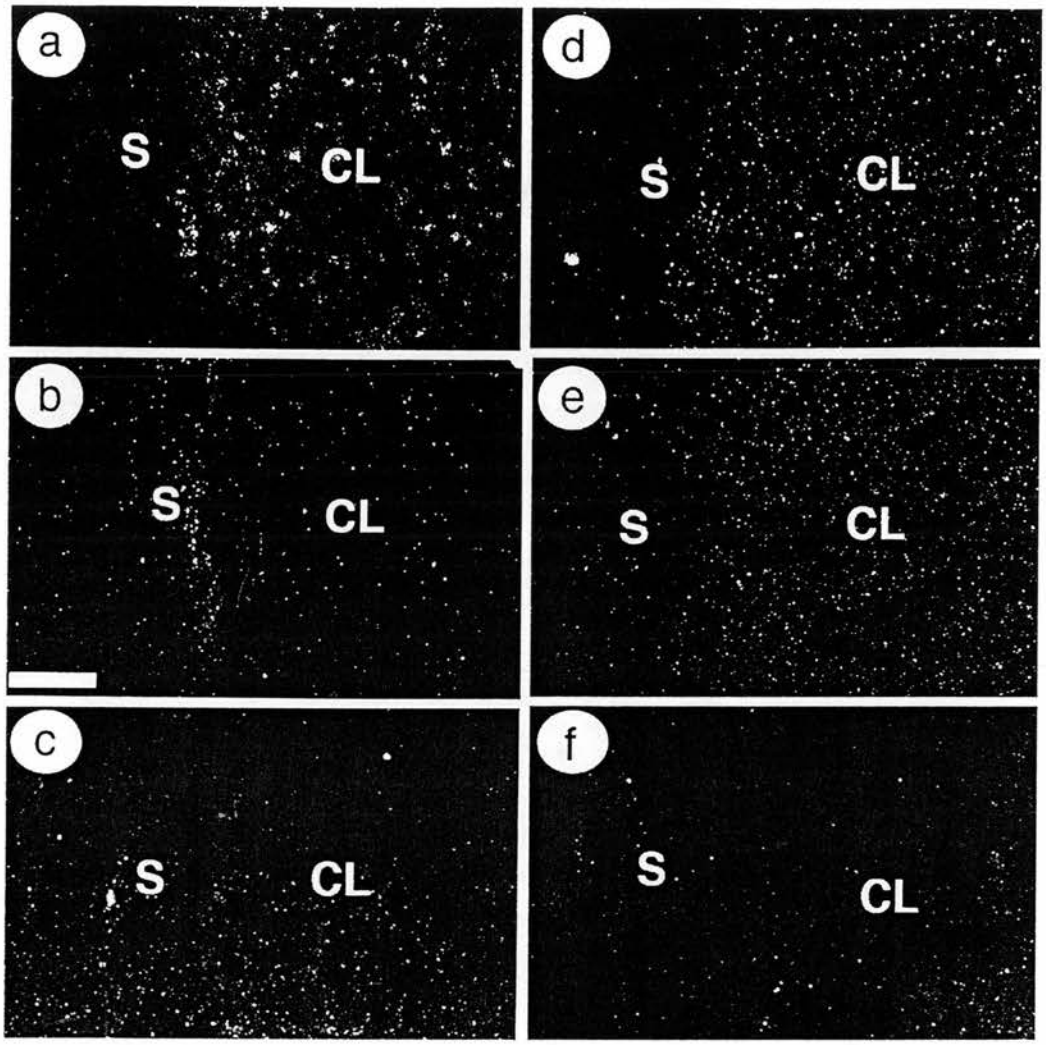

Figure 4. Luteinizing hormone (LH) receptor in the marmoset corpus luteum 12 and 24 h after induced luteolysis: (**a**) dark-field section of a mid-luteal marmoset ovary after in-situ hybridization for LH receptor mRNA showing signal in the corpus luteum (CL) but not in the surrounding stroma (S); (**b**) dark-field section of a marmoset ovary 12 h and (**c**) 24 h after luteolysis was induced with $GnRH_{ant}$ after in-situ hybridization for LH receptor mRNA showing no signal in the corpus luteum (CL) or stroma (S); (**d**) dark-field serial section of (**a**) showing LH binding in the corpus luteum (CL) but not the surrounding stroma (S); (**e**) dark-field serial section of (**b**), showing persistence of LH binding in the corpus luteum (CL) and its absence from the surrounding stroma (S); (**f**) dark-field serial section of (**c**) showing reduced specific LH binding in the corpus luteum. Scale bar = 100 μm.

3β-HSD after induced luteolysis

Both mRNA (Figure 5a) and protein (Figure 5b) for 3β-HSD could be detected in corpora lutea of mid-luteal phase ovaries. Twenty-four hours after induced luteolysis, with either $PGF_{2\alpha}$ or $GnRH_{ant}$ no mRNA for 3β-HSD could be detected in corpora lutea (Figure 5c) although it could still be detected in the theca cell layer of developing follicles. 24 h after induced luteolysis, 3β-HSD protein could still be detected by immunohistochemistry (Figure 5d), however, the immunostaining was more patchy and less intense (Figure 5d) in each ovary studied.

Discussion

This study reports the expression of the LH receptor and the steroidogenic enzyme 3β-HSD after induced luteolysis in the primate. We have previously reported the expression and localization of the LH receptor in the human corpus luteum throughout the functional luteal phase and during simulated early pregnancy (Duncan *et al.*, 1996a). In that study, it was not clear that LH receptor mRNA fell during the late-luteal phase. Other studies have suggested either an increase (Ravindranath *et al.*, 1992a) or a fall (Nishimori *et al.*, 1995; Minegishi *et al.*, 1997) in luteal LH receptor mRNA during the late-luteal phase in the primate. We therefore used a well established model (Fraser *et al.*, 1995a,b; Duncan *et al.*, 1996b) of induced luteolysis in the primate in this follow-up study, to investigate the effect of luteolysis of LH receptor expression. It is now clear that induced luteolysis in the primate is associated with a rapid loss of LH receptors from the corpus luteum.

Induced luteolysis has been shown to result in a fall in LH receptor expression in rodents (Bjurulf and Selstam, 1996) and ruminants (Guy *et al.*, 1995; Smith *et al.*, 1996). In these animals, $PGF_{2\alpha}$ is an important physiological luteolysin. In

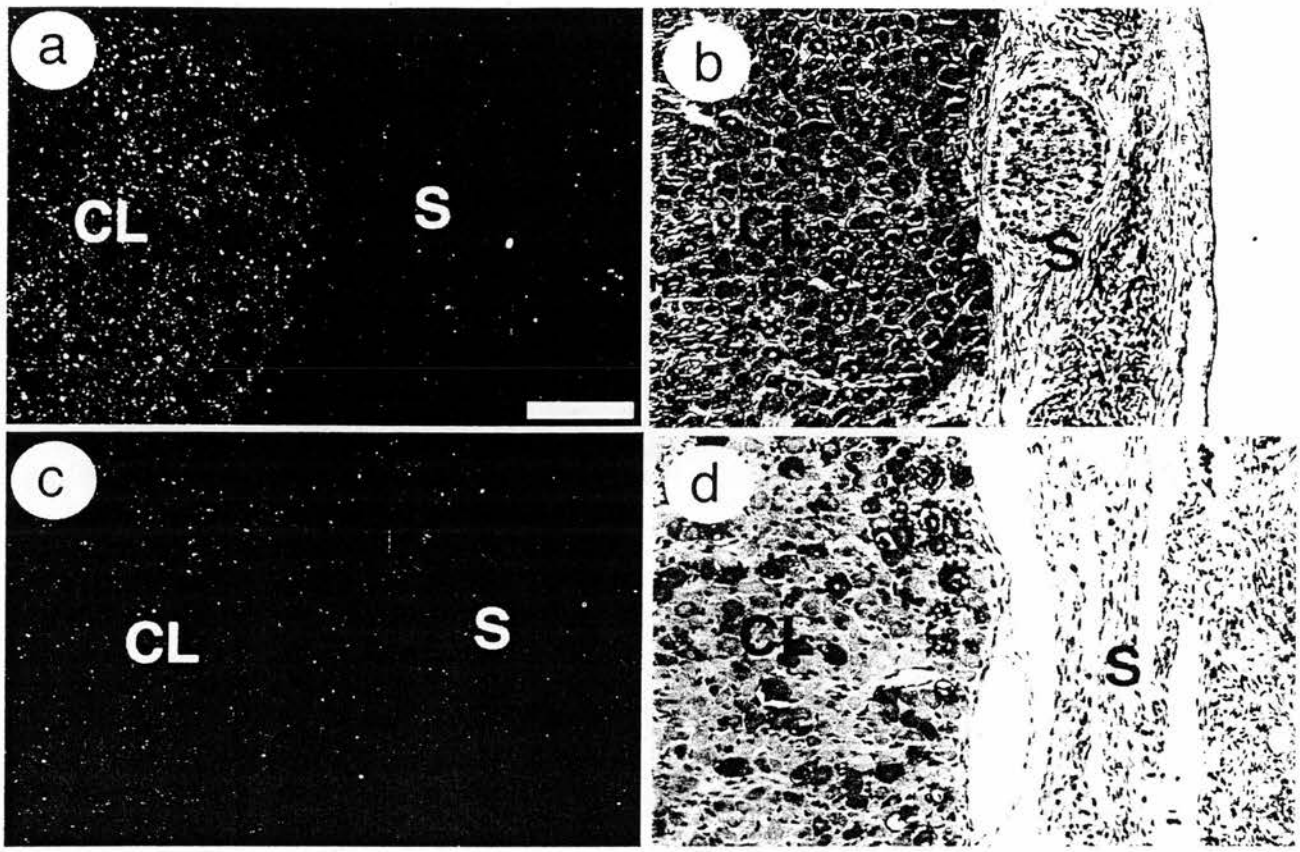

Figure 5. 3β-HSD in the marmoset corpus luteum after induced luteolysis: (**a**) dark-field section of a mid-luteal marmoset ovary after in-situ hybridization for 3β-HSD mRNA showing signal in the corpus luteum (CL) but not the surrounding stroma (S); (**b**) section of a mid-luteal marmoset ovary after immunohistochemistry for 3β-HSD showing staining of the corpus luteum (CL) but not the surrounding stroma (S); (**c**) dark-field section of a marmoset ovary 24 h after luteolysis was induced with $PGF_{2\alpha}$ after in-situ hybridization for 3β-HSD mRNA showing no signal in the corpus luteum (CL) and stroma (S); (**d**) section of a marmoset ovary 24 h after luteolysis was induced with $PGF_{2\alpha}$ after immunohistochemistry for 3β-HSD, showing patchy staining of the corpus luteum (CL) but not the surrounding stroma (S). Scale bar = 100 μm.

rats, luteal prostaglandin increases during the last days of the luteal phase (Olofsson *et al.*, 1990) and inhibition of prostaglandin synthesis by indomethacin prolongs the natural life-span of the corpus luteum (Bjurulf *et al.*, 1994). In sheep and cows, natural luteolysis is clearly attributed to the uterine synthesis and secretion of $PGF_{2\alpha}$ (Niswender *et al.*, 1985; Auletta and Flint, 1988). In these species, it is thought that $PGF_{2\alpha}$ has a direct effect on luteal LH receptor mRNA expression (Bjurulf and Selstam, 1996; Smith *et al.*, 1996). Although it is not clear in this study whether the reduced LH receptor mRNA is due to decreased transcription or decreased mRNA stability (Segaloff and Ascoli, 1993), it is clear that, despite different mechanisms of luteolysis in primates (Auletta and Flint, 1988), the effect of $PGF_{2\alpha}$ of LH receptor mRNA concentrations appears to be the same.

There is evidence that locally produced $PGF_{2\alpha}$ may have a role in primate luteolysis (Auletta *et al.*, 1984; Auletta and Flint, 1987; Behrman *et al.*, 1993). Prostaglandins are produced by the human corpus luteum and $PGF_{2\alpha}$ receptors can be detected in the human corpus luteum (Challis *et al.*, 1976). Pharmacological doses of prostaglandins can reduce progesterone secretion from the primate corpus luteum (Wentz and Jones, 1973; Auletta *et al.*, 1984). In addition, some studies have reported increased intra-luteal $PGF_{2\alpha}$ during the end of the functional human luteal phase (Shutt *et al.*, 1976; Patwardhan and Lanthier, 1980). As LH receptors cannot be detected in follicular phase primate corpora lutea (Ravandranath *et al.*, 1992a; Nishimori *et al.*, 1995; Takao *et al.*, 1997), it is clear that they disappear with functional luteolysis. Local $PGF_{2\alpha}$ may be involved in the inhibition of LH receptor expression at the end of the primate luteal phase.

The steroidogenic pathway can also be affected at other sites by $PGF_{2\alpha}$. *In vitro*, $PGF_{2\alpha}$ inhibits LH-stimulated progesterone production, and this is thought to be a post-cAMP-mediated effect (Auletta and Flint, 1988; Abayasekara *et al.*, 1993; Michael *et al.*, 1994). $PGF_{2\alpha}$ is known to activate protein kinase C (PKC) (Niswender *et al.*, 1994), which has several effects on the steroidogenic pathway. It inhibits cholesterol transport to cytochrome P450 side chain cleavage enzyme ($P450_{scc}$) (Wiltbank *et al.*, 1993), which suggests an inhibitory effect on sterol carrier protein-2 (SCP-2) (McLean *et al.*, 1995) or steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996). In addition it has been shown that expression of 3β-HSD can also be inhibited *in vitro* (Hawkens *et al.*, 1993). We have shown that 3β-HSD mRNA and protein expression are inhibited during $PGF_{2\alpha}$-induced luteolysis in the primate.

This confirms that the multiple sites of inhibition of steroidogenesis during $PGF_{2\alpha}$-induced luteolysis are also seen in the primate.

Induced luteolysis using LH withdrawal had the same effects on luteal LH receptor and 3β-HSD expression. It has previously been shown that removal of LH support in monkeys caused a dramatic down-regulation of mRNA for both $P450_{scc}$ and 3β-HSD (Ravindranath *et al.*, 1992b). Indeed, concentrations of 3β-HSD have been shown to decline during natural luteal regression in the primate (Doody *et al.*, 1990). We have confirmed the fall in 3β-HSD message and shown that this is associated with a fall in LH receptor mRNA. The similarity of the effects of the disparate ways to induce luteolysis in the marmoset suggests common final pathways of action. It is not known whether LH withdrawal induces local $PGF_{2\alpha}$ formation. However, it has been shown that HCG, acting through the LH receptor, has an inhibitory effect on luteolysis induced by $PGF_{2\alpha}$ (Auletta and Kelm, 1994). It is therefore likely that LH withdrawal and $PGF_{2\alpha}$ activate a common final pathway to induce luteolysis.

That common pathway might be progesterone withdrawal. It is clear that acute administration of $PGF_{2\alpha}$ can inhibit progesterone synthesis in the absence of changes in the expression of steroidogenic enzymes (Michael *et al.*, 1994). Its effects on cAMP stimulation and progesterone output precede the observed decrease in mRNA concentrations (Khan and Rosberg, 1979; Bjurulf and Selstam, 1996). Likewise, withdrawal of LH results in rapid cessation of progesterone output (Fraser *et al.*, 1986). It is possible that progesterone itself has an autocrine role in the corpus luteum. The primate corpus luteum possesses receptors to the progesterone it produces (Chandrasekher *et al.*, 1994; Suzuki *et al.*, 1994). Recent work using trilostane to inhibit progesterone synthesis suggests that progesterone may indeed have a major role in the function of the corpus luteum (Duffy *et al.*, 1994; Duffy and Stouffer, 1995). However, it is not yet clear if progesterone maintains the enzymes responsible for its production. Evidence from the rat, where $PGF_{2\alpha}$ only induces a transient decline in LH receptor and 3β-HSD mRNA suggests that their recovery occurs in the presence of follicular phase concentrations of progesterone (McLean *et al.*, 1995; Bjurulf and Selstam, 1996). The fact that we did not see this recovery, and that the rat does not appear to express luteal progesterone receptors (Parke-Sarge *et al.*, 1995) suggests that this may be a species effect. Progesterone therefore remains a potential candidate in the control of luteal function during luteolysis.

The effect of induced luteolysis on mRNA was evident before the effect on protein concentrations. We could detect little LH receptor and 3β-HSD mRNA 12 h after induced luteolysis. In sheep, Smith *et al.* (1996) reported that this reduction was evident within 6 h of induced luteolysis. We found continued LH receptor binding 12 h after induced luteolysis and could detect some 3β-HSD protein 24 h after induced luteolysis in the absence of mRNA. The patchy appearance of 3β-HSD we observed after induced luteolysis is similar to that seen during natural luteolysis in the monkey (Sanders and Stouffer, 1997). This time difference of effects on mRNA and protein concentrations was also seen in ovine corpora lutea after induced luteolysis with $PGF_{2\alpha}$ (Smith *et al.*, 1996). This is consistent with protein having a longer turnover time than mRNA. However, as progesterone concentrations were at follicular concentrations, 12 h after induced luteolysis, it appears that this protein is not stimulated enough to be functional at this stage. It therefore needs to be stressed that at the first time point studied (12 h), the progesterone concentrations were already at follicular concentrations. It is not clear whether the decline in progesterone precedes the inhibition of LH and 3β-HSD expression, is secondary to it, or related through another common factor. It would be interesting to dissect the pathway at time points earlier than 12 h.

Induced luteolysis in the marmoset monkey is associated with cell death and disruption of the cellular architecture (Fraser *et al.*, 1995a). Indeed it has been suggested that $PGF_{2\alpha}$ can directly cause apoptotic cell death (Sawyer *et al.*, 1990). It is possible that the fall in mRNA and protein for the LH receptor and 3β-HSD reflects a general loss of cell viability within the corpus luteum rather than being specific to functional luteolysis. This is unlikely, as the loss of mRNA clearly precedes the loss of protein for both the LH receptor and 3β-HSD, and other mRNA species are still present in the corpus luteum 12 h after induced luteolysis (Duncan *et al.*, 1996b). In addition, some proteins have been shown to increase in the corpus luteum after induced luteolysis in the marmoset (Woad *et al.*, 1996). This suggests that the steroidogenic pathway is specifically and rapidly switched off during induced luteolysis.

The rapid loss of LH receptor mRNA in the corpus luteum was not seen in the thecal layers of antral follicles after induced luteolysis. This LH receptor will be equally starved of its ligand after $GnRH_{ant}$-induced luteolysis. The inhibitory effects on LH receptor and 3β-HSD mRNA was clearly not seen in the thecal cells. This suggests that the common luteolytic pathway is not found in follicular thecal cells. It is not known whether these cells express $PGF_{2\alpha}$ receptors. In the sheep corpus luteum, $PGF_{2\alpha}$ receptors are located on the large luteal cells, and not the small luteal cells, that are thought to be of thecal origin (Fitz *et al.*, 1982). This may be one of the differences. Thecal cells of the follicle, however, may express progesterone receptors (Suzuki *et al.*, 1994). Clearly studying the differences between follicular thecal cells and luteal cells may aid understanding of the luteolytic process.

In conclusion, the rapid reduction of LH receptors and the steroidogenic enzyme 3β-HSD during induced luteolysis in the primate suggests an inhibition to synthesis of the components of the steroidogenic pathway as well as their function during luteolysis. It is still not clear if this is a cause or effect of low progesterone concentrations, but it is likely to contribute to the continued inhibition of progesterone synthesis during luteolysis. The similarity of effect of $PGF_{2\alpha}$ treatment and LH withdrawal, however, suggests a common inhibitory pathway. Dissection of this pathway may give more information about the continuing enigma of primate luteolysis.

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References

Abayasekara, D.R.E., Michael, A.E., Webley, G.E. and Flint, A.P.F. (1993) Mode of action of prostaglandin $F_{2\alpha}$ in human luteinized granulosa cells: role of protein kinase C. *Mol. Cell. Endocrinol.*, **97,** 81–91.

Auletta, F.J. and Flint, A.P.F. (1988) Mechanisms controlling corpus luteum function in sheep, cows, nonhuman primates, and women especially in relation to the time of luteolysis. *Endocrine Rev.*, **9,** 88–105.

Auletta, F.J. and Kelm, L.B. (1994) Mechanisms controlling corpus luteum function in the rhesus monkey (*Macaca mulatta*): inhibitory action of hCG on luteolysis induced by $PGF_{2\alpha}$. *J. Reprod. Fertil.*, **102,** 215–220.

Auletta, F.J., Kamps, D.L., Wesley, M. and Gibson, M. (1984) Luteolysis in the rhesus monkey: ovarian venous estrogen, progesterone, and prostaglandin $F_{2\alpha}$-metabolite. *Prostaglandins*, **27,** 299–310.

Behrman, H.R., Endo, T., Aten, R.F. and Musicki, B. (1993) Corpus luteum function and regression. *Reprod. Med. Rev.*, **2,** 153–180.

Bjurulf, E. and Selstam, G. (1996) Rat luteinizing hormone receptor messenger ribonucleic acid expression and luteolysis: inhibition by prostaglandin $F_{2\alpha}$. *Biol. Reprod.*, **54,** 1350–1355.

Bjurulf, E., Selstam, G. and Olofsson, J.L. (1994) Increased LH receptor mRNA and extended corpus luteum function induced by prolactin and indomethacin treatment *in vitro* in hysterectomized pseudopregnant rats. *J. Reprod. Fertil.*, **102,** 139–145.

Challis, J.R.G., Calder, A.A., Dilley, S. *et al.* (1976) Production of prostaglandins E and F by corpora lutea, corpora albicantes and stroma from the human ovary. *J. Endocrinol.*, **68,** 401–408.

Chandrasekher, Y.A., Melner, M.H., Nagalla, S.R. and Stouffer, R.L. (1994) Progesterone receptor, but not estradiol receptor, messenger ribonucleic acid is expressed in luteinizing granulosa cells and the corpus luteum in rhesus monkeys. *Endocrinology*, **135,** 307–314.

Deghenghi, R., Boutignon, F., Wuthrich, P. and Lenaerts, V. (1993) Antarelix (EP 24332) a novel water soluble LHRH antagonist. *Biomed. Pharmacol.*, **47,** 107–110.

Doody, K.J., Lorence, M.C., Mason, J.I. and Simpson, E.R. (1990) Expression of messenger ribonucleic acid species encoding steroidogenic enzymes in human follicles and corpora lutea throughout the menstrual cycle. *J. Clin. Endocrinol. Metab.*, **70,** 1041–1045.

Duffy, D.M. and Stouffer, R.L. (1995) Progesterone receptor messenger ribonucleic acid in the primate corpus luteum during the menstrual cycle: possible regulation by progesterone. *Endocrinol.*, **136,** 1869–1876.

Duffy, D.M., Hess, D.L. and Stouffe, R.L. (1994) Acute administration of a 3β-hydroxysteroid dehydrogenase inhibitor to rhesus monkeys at the mid-luteal phase of the menstrual cycle: evidence for possible autocrine regulation of the primate corpus luteum by progesterone. *J. Clin. Endocrinol. Metab.*, **79,** 1587–1594.

Duncan, W.C., McNeilly, A.S., Fraser, H.M. and Illingworth, P.J. (1996a) Luteinizing hormone receptor in the human corpus luteum: lack of down-regulation during maternal recognition of pregnancy. *Hum. Reprod.*, **11,** 2291–2297.

Duncan, W.C., Illingworth, P.J. and Fraser, H.M. (1996b) Expression of tissue inhibitor of metalloproteinases-1 in the primate ovary during induced luteal regression. *J. Endocrinol.*, **151,** 203–213.

Endo, T., Aten, R.F., Wang, F. and Behrman, H.R. (1993) Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. *Endocrinology*, **133,** 690–698.

Fitz, T.A., Mayan, M.H., Sawyer, H.R. and Niswender, G.D. (1982) Characterization of two steroidogenic cell types in the ovine corpus luteum. *Biol. Reprod.*, **27,** 703–711.

Fraser, H.M., Abbott, M., Laird, N.C. *et al.* (1986) Effects of an LHRH antagonist on the secretion of LH, FSH, prolactin and ovarian steroids at different stages of the luteal phase in the stumptailed *Macacca arctoides*. *J. Endocrinol.*, **111,** 83–89.

Fraser, H.M., Lunn, S.F., Cowen, G.M. and Illingworth, P.J. (1995a) Induced luteal regression in the primate: evidence for apoptosis and changes in *c-myc* protein. *J. Endocrinol.*, **147,** 131–137.

Fraser, H.M., Lunn, S.F., Whitelaw, P.F. and Hillier, S.G. (1995b) Induced luteal regression: differential effects on follicular and luteal inhibin/activin subunit mRNAs in the marmoset monkey. *J. Endocrinol.*, **144,** 201–208.

Guy, M.K., Juengel, J.L., Tandeski, T.R. and Niswender, G.D. (1995) Steady-state concentrations of messenger RNA encoding the receptor for luteinizing hormone during the estrous cycle and following prostaglandin F2 alpha treatment of ewes. *Endocrine*, **3,** 585–589.

Hawkens, D.E., Belfiore, C.J., Kile, J.P. and Niswender, G.D. (1993) Regulation of messenger ribonucleic acid encoding 3β-hydroxysteroid dehydrogenase/D^5-D^4-isomerase in the ovine corpus luteum. *Biol. Reprod.*, **48,** 362–366.

Hutchison, J.S. and Zeleznik, A.J. (1985) The corpus luteum of the primate menstrual cycle is capable of recovering from a transient withdrawal of pituitary gonadotropin support. *Endocrinology*, **117,** 1043–1049.

Juengel, J.L., Garverick, H.A., Johnson, A.L. *et al.* (1993) Apoptosis during luteal regression in cattle. *Endocrinology*, **132,** 249–254.

Khan, M.I. and Rosberg, S. (1979) Acute suppression by $PGF_{2\alpha}$ on LH, epinephrine and fluoride stimulation of adenylate cyclase in rat luteal tissue. *J. Cyclic Nucleotide Res.*, **5,** 55–63.

McLean, M.P., Billheimer, J.T., Warden, K.J. and Irby, R.B. (1995) Prostaglandin $F_{2\alpha}$ mediates ovarian sterol carrier protein-2 expression during luteolysis. *Endocrinology*, **136,** 4963–4972.

Michael, A.E., Abayasekara, D.R.E. and Webley, G.E. (1994) Cellular mechanisms of luteolysis. *Mol. Cell. Endocrinol.*, **99,** R1–R9.

Minegishi, T., Tano, M., Abe, Y. *et al.* (1997) Expression of luteinizing hormone/human chorionic gonadotrophin (LH/HCG) receptor mRNA in the human ovary. *Mol. Hum. Reprod.*, **3,** 101–107.

Nishimori, K., Dunkel, L., Hsueh, A.J.W. *et al.* (1995) Expression of luteinizing hormone and chorionic gonadotropin receptor messenger ribonucleic acid in human corpora lutea during menstrual cycle and pregnancy. *J. Clin. Endocrinol. Metab.*, **80,** 1444–1448.

Niswender, G.D., Schwall, R.H., Fitz, T.A. *et al.* (1985) Regulation of luteal function in domestic ruminants: new concepts. *Rec. Prog. Hormone Res.*, **41,** 101–151.

Niswender, G.D., Juengel, J.L., McGuire, W.J. *et al.* (1994) Luteal function: the estrous cycle and early pregnancy. *Biol. Reprod.*, **50,** 239–247.

Olofsson, J., Norjavaara, E. and Selstam, G. (1990) *In vivo* levels of prostaglandin $F_{2\alpha}$, E_2 and prostacyclin in the corpus luteum of pregnant and pseudopregnant rats. *Biol. Reprod.*, **42,** 792–768.

Parke-Sarge, O.-K., Parmer, T.G., Gu, Y. and Gibori, G. (1995) Does the rat corpus luteum express the progesterone receptor gene? *Endocrinology*, **136,** 1537–1543.

Patwardhan, V.V. and Lanthier, A. (1980) Concentration of prostaglandins, PGE, PGF, estrone, estradiol, and progesterone in human corpora lutea. *Prostaglandins*, **20,** 963–969.

Ravindranath, N., Little-Ihrig, L.L. and Zeleznik, A.J. (1992a) Characterization of the levels of messenger ribonucleic acid that encode for luteinizing hormone receptor during the luteal phase of the primate menstrual cycle. *J. Clin. Endocrinol. Metab.*, **74,** 779–785.

Ravindranath, N., Little-Ihrig, L.L., Benyo, D.F. and Zeleznik, A.J. (1992b) Role of luteinizing hormone in the expression of cholesterol side-chain cleavage cytochrome P450 and 3β-hydroxysteroid dehydrogenase in the primate corpus luteum. *Endocrinology*, **131,** 2065–2070.

Sanders, S.L. and Stouffer, R.L. (1997) Localization of steroidogenic enzymes in macaque luteal tissue during the menstrual cycle and simulated early pregnancy: immunohistochemical evidence supporting the two-cell model for estrogen production in the primate corpus luteum. *Biol. Reprod.*, **56,** 1077–1087.

Sawyer, H.R., Niswender, K.D., Braden, T.D. and Niswender, G.D. (1990) Nuclear changes in ovine luteal cells in response to $PGF_{2\alpha}$. *Domest. Anim. Endocrinol.*, **7,** 229–238.

Segaloff, D.L. and Ascoli, M. (1993) The lutropin/choriogonadotropin receptor... 4 years later. *Endocrine Rev.*, **14,** 324–347.

Shutt, D.A., Clark, A.H., Fraser, I.S. *et al.* (1976) Changes in concentration of prostaglandin F and steroids in human corpora lutea in relation to growth of the corpus luteum and luteolysis. *J. Endocrinol.*, **71,** 453–454.

Smith, G.W., Gentry, P.C., Roberts, R.M. and Smith, M.F. (1996) Ontogeny and regulation of luteinizing hormone receptor messenger ribonucleic acid within the ovine corpus luteum. *Biol. Reprod.*, **54,** 76–83.

Smith, K.B., Lunn, S.F. and Fraser, H.M. (1990) Inhibin secretion during the ovulatory cycle and pregnancy in the common marmoset monkey. *J. Endocrinol.*, **126,** 489–495.

Stocco, D.M. and Clark, B.J. (1996) Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Rev.*, **17,** 221–244.

Stouffer, R.L., Gulyas, B.J., Nixon, W.E. and Hodgen, G.D. (1977) Gonadotropin-sensitive progesterone production by rhesus monkey luteal cells *in vitro*: a function of the age of the corpus luteum during the menstrual cycle. *Endocrinology*, **100,** 506–512.

Suzuki, T., Sasano, H., Kimura, N. *et al.* (1994) Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: relationship to expression of steroidogenic enzymes. *Hum. Reprod.*, **9,** 1589–1595.

Takao, Y., Honda, T., Ueda, M. *et al.* (1997) Immunohistochemical localization of the LH/HCG receptor in human ovary: HCG enhances cell surface expression of LH/HCG receptor on luteinizing granulosa cells *in vitro*. *Mol. Hum. Reprod.*, **3,** 569–578.

Wentz, A.C. and Jones, G.S. (1973) Transient luteolytic effect of prostaglandin $F_{2\alpha}$ in the human. *Obstet. Gynecol.*, **42,** 172–181.

Wiltbank, M.C., Belfiore, C.J. and Niswender, G.D. (1993) Steroidogenic enzyme activity after acute activation of protein kinase (PK) A and PKC in ovine small and large luteal cells. *Mol. Cell. Endocrinol.*, **97,** 1–7.

Woad, K.J., Norman, B. and Fraser, H.M. (1996) Immunohistochemical localisation of clusterin during luteolysis in the primate. *J. Reprod. Fertil.*, (Abstract series), **17,** 59.

Young, F.M., Illingworth, P.J., Lunn, S.F. *et al.* (1997) Cell death during luteal regression in the marmoset monkey (*Callithrix jacchus*). *J. Reprod. Fertil.*, **111,** 109–119.

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Immunolocalization of bcl-2 protein in human endometrium in the menstrual cycle and simulated early pregnancy

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Cell death by apoptosis is now regarded as an important feature of normal endometrial physiology. Recent reports have suggested that *bcl-2*, a proto-oncogene responsible for the suppression of apoptosis, is expressed in endometrium and may be involved in the regulation of menstruation. Using standard immunohistochemical procedures, the immunoreactivity of bcl-2 and progesterone receptors has been investigated in normal human endometrium throughout the menstrual cycle (n = 25) as well as endometrium exposed to continued oestradiol and progesterone stimulation by 'rescue' of corpus luteum (n = 4) with exogenous human chorionic gonadotrophin (HCG) administration (pseudopregnancy). Marked immunoreactivity, consistent with previous reports, was noted in the glandular epithelium during the proliferative phase of the cycle. Immunostaining persisted in the glandular epithelium during the secretory phase, although the percentage and intensity of staining was markedly reduced. Staining in the stromal compartment was only noted during the late secretory phase of the cycle. Co-localization with an antibody against CD56 demonstrated that this immunoactivity largely reflected the presence of lymphocytes in the stroma. Endometrium from subjects who underwent 'luteal rescue' displayed limited immunostaining in either glands or stroma. The absence of significant *bcl-2* expression in endocrinologically maintained endometrium makes it highly unlikely that *bcl-2* is important in prolonging endometrial cell survival in the luteal phase of the menstrual cycle.

Key words: apoptosis/bcl-2/endometrium/immunohistochemistry/pseudopregnancy

Introduction

Apoptosis is a distinct mode of cell death that is responsible for deletion of cells in normal tissues (Kerr *et al.*, 1972). This process is quite distinct from cell death in response to toxic effects (necrosis) and is characterized by specific morphological features such as cell shrinkage, nuclear chromatin condensation and fragmentation leading to the formation of membrane-enclosed apoptotic bodies containing well-preserved organelles (Kerr *et al.*, 1994). Apoptosis has been noted to be involved in the involution of some endocrine-dependent target organs such as the adrenal gland (Wyllie *et al.*, 1973), the ovary (Hurwitz and Adashi, 1992) and the prostate (Kyprianou and Isaacs, 1988). Apoptosis has also been reported in rabbit endometrium after withdrawal of progesterone (Nawaz *et al.*, 1987) as well as human endometrium at the end of the luteal phase (Hopwood and Levison, 1976). In addition to the detailed morphological features of apoptosis, specific nuclear DNA fragmentation has been demonstrated in human endometrium by 3′ end-labelling techniques (Tabibzadeh *et al.*, 1994)

Apoptosis is known to be controlled by the expression of a number of regulatory genes, including c-*myc*, *p53*, *apo-1/fas* and particularly members of the expanding *bcl-2* family. Bcl-2, the product of a proto-oncogene, is localized in the inner mitochondrial membrane (Hockenbery *et al.*, 1990) and has been shown to prolong cell survival by suppressing apoptosis in various cells (Vaux *et al.*, 1992; Baer, 1994). Two recent reports have described the expression of *bcl-2* in normal human endometrium (Gompel *et al.*, 1994; Otsuki *et al.*, 1994). Both reports observe that the level of expression in the glandular cells is maximal during the proliferative phase of the menstrual cycle but while one report (Otsuki *et al.*, 1994) suggests that bcl-2 is present during the early part of the luteal phase, the other (Gompel *et al.*, 1994) found that bcl-2 disappeared from the endometrium 2–3 days after the onset of secretory change, with only weak immunoreactivity being evident later in the luteal phase.

Only Gompel *et al.* (1994) made detailed reference to the pattern of stromal immunoreactivity. In the proliferative phase, stromal staining was scarce with only the occasional lymphocyte (positive internal control) displaying positive immunoreactivity. Stromal staining increased with predecidualization of the stromal compartment and in association with the increase in the presence of lymphocytes. However, the cell types expressing bcl-2 were not characterized. This is important, as bcl-2 plays a central role in lymphocyte selection in response to an antigenic stimulus (Cohen and Duke, 1992). As it

is likely that processes such as vasoconstriction in response to endothelins or prostaglandins are more important than glandular apoptosis *per se* in the turnover of the endometrium, it is possible that stromal expression of bcl-2 may represent an altogether different process.

From these data, it is unclear what role bcl-2 plays (if any) in the processes leading up to menstruation in the non-pregnant cycle or establishment of pregnancy in the pregnant cycle. To investigate this further, we have studied the expression of bcl-2 in carefully timed endometrial biopsies as well as biopsies from women in whom the endocrinology of early pregnancy has been simulated by injections of exogenous human chorionic gonadotrophin (HCG). We have particularly investigated the nature of the previously reported bcl-2 positive cells in the stroma of the endometrium.

Materials and methods

Samples of endometrial tissue were collected from 29 women aged between 25 and 45 years. Samples were either collected from the uterine specimens of women undergoing elective hysterectomy or by endometrial biopsy from women undergoing laparoscopic sterilization. All subjects described regular menstrual cycles of between 25 and 35 days and were of proven fertility. No subject had received any drug or hormonal therapy during the 3 months previous to the onset of their last menstrual period.

The subjects were grouped according to the stage of the menstrual cycle as follows: early proliferative phase (days 4–9 after onset of menses, $n = 5$); late proliferative phase (days 10–14 after onset of menses, $n = 3$); early secretory phase (days 1–4 after ovulation, $n = 5$); mid-secretory phase (days 5–8 after ovulation, $n = 3$); late secretory phase (days 11–14 after ovulation, $n = 8$). Eighteen of the subjects whose operation took place during the luteal phase of the menstrual cycle had collected daily urine samples throughout the cycle of operation. The luteinizing hormone (LH) concentration was subsequently assayed in these samples and the day of ovulation taken as the day of the urinary LH peak (Djahanbakhch *et al.*, 1981a). Progesterone concentration was measured in these subjects by standard radioimmunoassay (Djahanbakhch *et al.*, 1981b) in a single plasma sample collected on the morning of the operation and expressed as mean ± SE. The endocrine dating was supported by histological dating on haematoxylin and eosin sections according to the criteria of Noyes *et al.* (1950).

Four subjects, where the operation was due to fall between 12 and 16 days after ovulation, agreed to receive HCG (Profasi, Serono) to maintain the lifespan of the corpus luteum. The HCG was administered by i.m. injection for between 6 and 8 days in incremental doses beginning with 125 IU per day then doubling daily to a maximum of 16 000 IU per day. Plasma samples were collected on alternate days during HCG administration. The concentrations of HCG as well as the ovarian hormones, oestradiol, progesterone and inhibin produced by this regime are similar to those seen in normal pregnancy (Illingworth *et al.*, 1990).

All tissue specimens were fixed in either 10% neutral buffered formalin or 4% paraformaldehyde (the method of fixation did not affect bcl-2 immunoreactivity) and thereafter routinely embedded in paraffin wax. Tissue sections (5 μm) were cut, dried overnight at 55°C and processed for (i) haematoxylin and eosin staining for routine histological dating, (ii) immunohistochemistry for expression of bcl-2 protein, progesterone receptor and CD56 antigen.

Immunohistochemistry

bcl-2

The bcl-2 protein was immunolocalized with a monoclonal mouse antibody (bcl-2–124, Dako Ltd, High Wycombe, Bucks, UK) raised against a synthetic peptide sequence comprising amino acids 41–54 of bcl-2 protein (Tsujimoto and Croce, 1986). The immunohistochemical detection of bcl-2 was conducted on de-paraffinized sections using an avidin biotin peroxidase detection system (Vector Laboratories, Peterborough, UK). Briefly, sections were de-paraffinized and rehydrated through graded alcohols, followed by microwaving at high power in 0.01 M sodium citrate buffer (pH 6) for 15 min. Endogenous peroxidase immunostaining was blocked by treatment with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Thereafter, non-specific binding was blocked by incubation with normal rabbit serum. Tissue sections were then successively incubated with anti-human bcl-2 mouse monoclonal antibody for 60 min at 37°C, then with a horse biotinylated anti-mouse antibody (Vector Laboratories, Peterborough, UK), and finally with an avidin–biotin peroxidase complex (ABC, Vector Laboratories, Peterborough, UK). Sections were incubated with diaminobenzidine (DAB) and lightly counterstained with haematoxylin prior to permanent mounting. Tonsil tissue was included as a positive control. As negative controls, mouse immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used.

Progesterone receptor (PR)

A monoclonal mouse antibody, raised against a synthetic peptide towards the amino end of the human PR molecule, was used to immunolocalize the PR (PGR Paraffin, Novocastra Laboratories, Newcastle, UK). This antibody recognizes both A and B forms of receptor as well as bound and unbound receptor. PR was immunolocalized using the avidin–biotin peroxidase detection system described above using a 1:20 dilution of the primary antibody (0.6 μg/ml). Normal goat serum (SAPU, Carluke, Lanarkshire, UK) was used to block non-specific binding and a 1:100 dilution of biotinylated goat anti-mouse (Dako Ltd, High Wycombe, Bucks, UK) was used as the secondary antibody. As PR is a nuclear protein, these sections were not counterstained with haematoxylin.

CD56

CD56 positive cells were immunolocalized with a mouse monoclonal antibody (Zymed Laboratories, San Francisco, CA, USA). Primary antibody was used at a dilution of 1:250. As a negative control, the primary antibody was replaced with mouse immunoglobulin of the same concentration. An avidin biotin peroxidase detection system was employed (Vector

Laboratories, Peterborough, UK) and V.I.P. chromogen (Vector Laboratories, Peterborough, UK)

Image analysis

Bcl-2

Immunoreactivity was assessed using light microscopy and the intensity of immunostaining for bcl-2 in all tissue sections was semi-quantitatively scored on a five point scale where 0 = no staining, 1 = mild staining, 2 = moderate staining, 3 = intense staining and 4 = very intense staining. The percentage of positively stained cells in each tissue section was estimated in the gland, stromal and surface epithelial compartments.

Progesterone receptor

Computer-aided nuclear densitometry was performed on sections from the same carefully controlled experimental run. Grey-scale densitometry was performed after image capture using an image analysis program (Image 1.52, NIH, Maryland, USA) on a Macintosh IICX computer. The average grey-scale reading of 50 random nuclei in three fields of view was calculated for the nuclei of the glands and stroma on each section. Background readings (taken from the cytoplasm of the same cells) were subtracted from the average grey-scale reading to give a more objective measurement of staining intensity in both stromal and glandular tissue.

Informed consent was obtained from all volunteers. Ethical approval for this study was granted by the Lothian Ethics of Medical Research Committee, Paediatric and Reproductive Medicine Subcommittee.

Results

Endocrinology

In all women who received HCG, menses was postponed. The mean progesterone concentrations in the luteal phase subjects were: early 16.77 ± 4.28 nmol/l; mid 34.04 ±6.73 nmol/l; late 28.01±7.37 nmol/l; HCG treated, 54.76±2.45 nmol/l.

Positive control (tonsil)

Intense brown staining for bcl-2 protein was observed in the follicular mantle and the majority of cells within the germinal centre of the tonsil displayed negative immunostaining (Figure 1a). The negative control (primary antibody replaced with normal mouse immunoglobulin) showed an absence of specific staining in the follicular mantle. In tonsil tissue and all endometrial biopsies, bcl-2 positive immunostaining was consistently cytoplasmic.

Proliferative phase endometrium

Positive immunostaining for bcl-2 protein was observed in the glandular cells of proliferative phase endometrium. The stroma and surface epithelium occasionally also displayed positive immunoreactivity (Figure 1b). Immunoreactivity was most marked in mid- and late proliferative phase glandular epithelium (Figure 2a). Stromal immunoreactivity was minimal with occasional isolated cells (presumed lymphocytes) displaying positive immunoreactivity (Figure 2b). A serial section, where the primary antibody had been replaced with normal mouse immunoglobulin, displayed a complete absence of immunostaining in all cells (Figure 1c).

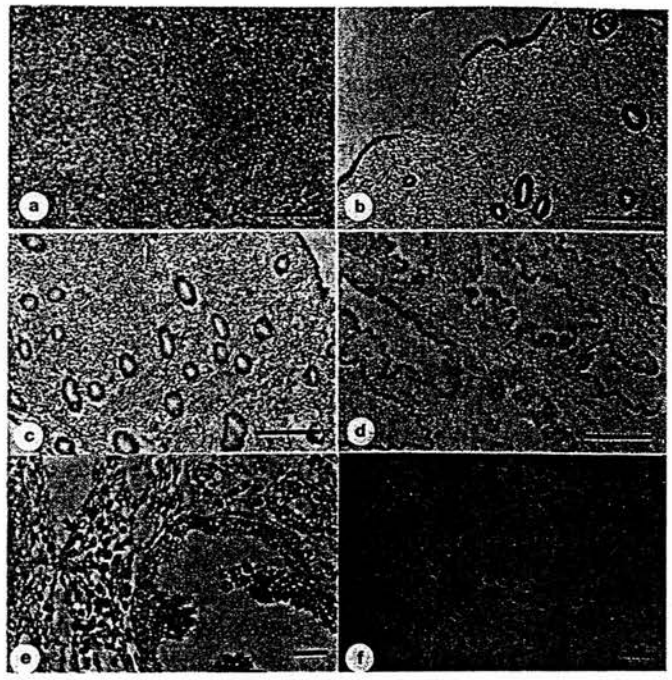

Figure 1. (**a**) Immunoperoxidase staining (brown reaction product) for bcl-2 in human tonsil, scale bar = 100 μm; (**b**) immunolocalization of bcl-2 protein in mid-proliferative endometrium, scale bar = 200 μm; (**c**) negative control, note the absence of specific bcl-2 immunoreactivity, scale bar = 200 μm; (**d**) late secretory phase endometrium displaying marked immunoreactivity in glandular epithelium and also isolated stromal cells, scale bar = 200 μm; (**e**) late secretory phase endometrium showing co-localization of bcl-2 protein (brown) in stromal cells and CD56 antigen (purple, arrows), scale bar = 25 μm; (**f**) HCG rescued endometrium (pseudopregnant) displaying nuclear (arrow) immunoreactivity for progesterone receptor (brown) in the stromal compartment (note the absence of glandular staining), scale bar = 25 μm.

Secretory endometrium

The early secretory phase endometrium displayed weak immunoreactivity in both glandular and surface epithelium with infrequent stromal positive immunoreactivity. Both the intensity of staining in the glandular epithelium and the percentage of cells stained were reduced in the luteal phase endometrium (Figure 1d,e). The intensity of staining was very variable (Figure 2a) but in late secretory phase endometrium, bcl-2 immunostaining was noted to be higher in both glandular and surface epithelium (data not shown). The mean percentage of cells exhibiting positive staining for bcl-2 was 9% in the early luteal phase, 13% in the mid-luteal phase and 36% of cells in the late luteal phase. The late secretory biopsies were the only sections to show high levels of immunoreactivity in the stromal compartments (Figure 2b).

Co-localization with CD56 showed that many, although not all, of the cells in the stromal compartment that were bcl-2 positive were also positive for CD56. In particular, lymphoid aggregates close to the gland were positive for both CD56 and

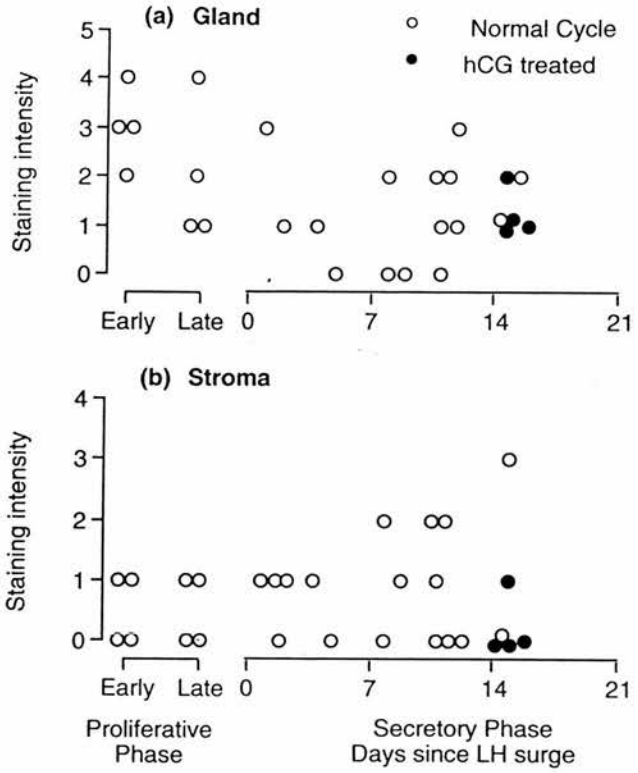

Figure 2. Density of staining for bcl-2 in proliferative and secretory phase endometrium (density estimated by arbitrary scoring system), plotted against the number of days since the urinary luteinizing hormone (LH) surge in (**a**) glandular tissue and (**b**) stroma.

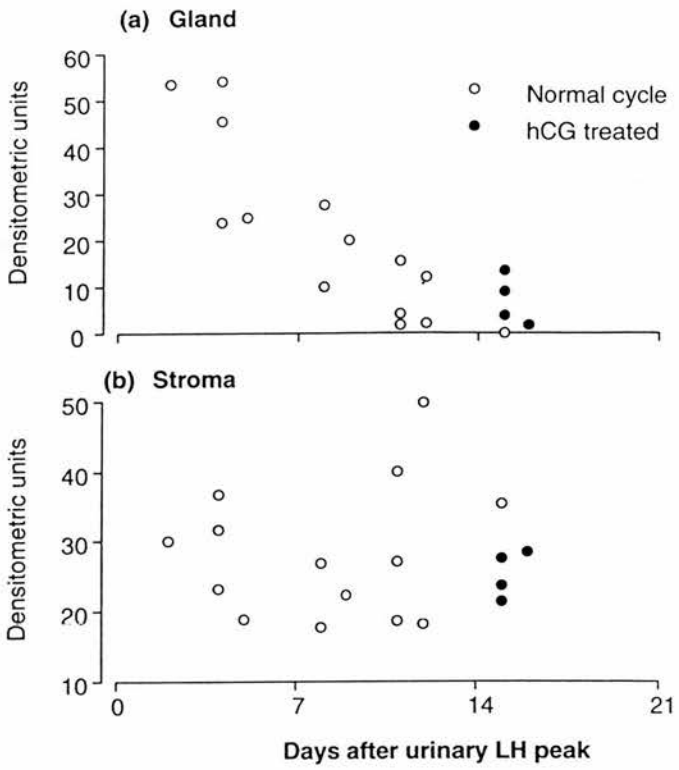

Figure 3. Immunolocalization of progesterone receptors (arbitrary densitometric units) in secretory phase endometrium, plotted against the number of days since the urinary LH surge in (**a**) glandular tissue and (**b**) stroma.

bcl-2 (Figure 1e). The immunoreactivity for the PR in the glandular epithelium present in the early secretory phase is not present in the mid- or late secretory phase as shown in Figure 3a. There are no differences in the stromal immunoreactivity for the PR in the early, mid- and late secretory phases (Figure 3b).

HCG rescued endometrium (pseudo-pregnancy)

The morphology of these glands was found to be very similar to that observed in the mid-luteal phase sections with tortuous glands exhibiting intraluminal secretion with loss of blebbing. There was little morphological evidence of apoptosis in these sections and no pre-decidual changes were evident. In one of the subjects who had received HCG for 8 days, the Arias–Stella phenomenon of pregnancy was evident.

Weak immunostaining for bcl-2 was found in a small proportion of glandular cells. The level of immunostaining in the stromal cells was similarly very limited in comparison with the late luteal phase. These cells were also positive for CD56. The pattern of localization of PR in pseudo-pregnant endometrium shows a very similar pattern to that of the mid and late secretory endometrium (Figures 1f, 3a,b).

Discussion

The present study supports the findings of previous studies (Gompel *et al.*, 1994; Otsuki *et al.*, 1994) that *bcl-2* is expressed in the glandular component of human endometrium. The finding in this study of maximal staining intensity during the proliferative phase is entirely consistent with these earlier reports. There was, however, a marked discrepancy between the luteal phase observations of our study and those of the two previous studies: Otsuki *et al.* (1994) finding very little immunostaining for bcl-2 at any stage of the luteal phase while Gompel *et al.* (1994) noted staining only in the early luteal phase. In contrast, we observed staining in the glandular component of endometrial samples from all stages of the luteal phase, including the late luteal phase, although the pattern and intensity of staining was very variable. Whether these discrepancies in luteal phase are a reflection of endometrial dysfunction (18 of the luteal phase subjects were undergoing hysterectomy for abnormally heavy periods) is not clear. However, the study of endometrial samples obtained from women where the time of ovulation has been clearly defined by serial urine estimation enables us more clearly to identify the chronology of events towards the end of the luteal phase.

The pattern of staining seen in the stromal compartment of the endometrium is entirely different from that seen in the glandular compartment. Significant immunoreactivity was absent in the proliferative phase and early secretory phase and became evident only in the late luteal phase. Indeed, as the time since ovulation increased so did the percentage of cells in the endometrium exhibiting bcl-2 staining. Our observation that most of these cells are CD56 positive lymphocytes suggests that the bcl-2 immunoreactivity seen in this part of the gland may represent the presence of immune cells which have been selected for survival. The survival of these immune cells may

be necessary for the secretion of cytokines such as IL-8 and tumour necrosis factor-α (TNFα) to initiate the processes leading to menstruation (Kelly, 1994).

Indeed we found that where the women had been treated with increasing doses of HCG, the detectable immunoactivity for bcl-2 was low. Treatment with HCG simulates pregnancy in that progesterone concentrations are maintained (as in all subjects studied here), although clearly may differ due to the absence of cytokines and growth factors of embryonic origin. In all cases, the process of menstruation has been inhibited, and although the histological appearances of this tissue show no clear evidence of decidualization this may simply reflect the short duration of exposure to HCG/progesterone. The model therefore enables us to compare the expression of bcl-2 in sections where the progesterone concentration is falling prior to menstruation with those where the progesterone concentration has been maintained. We found that in both the stroma and the glandular compartments, the proportion of cells expressing bcl-2 was reduced in these sections. This argues strongly against the hypothesis advanced by Otsuki *et al.* (1994) that bcl-2 has an important role in regulating death of glandular cells in the functional layer during the late luteal phase.

These observations are entirely consistent with the view that the expression of bcl-2 protein in the epithelium is under steroid control, in that oestrogen up-regulated expression while in the sections where progesterone concentrations were maintained, progesterone exposure down-regulated expression. However, the pattern of persistent immunostaining in some sections where progesterone concentrations are elevated implies that the regulation of endometrial bcl-2 expression may be the result of a combination of endocrine and paracrine signals. Tabibzadeh (1994) has suggested that T-cells and the cytokine TNFα may be implicated in the regulation of apoptosis of epithelial cells. In addition to T-cells, other cells in the stroma and endometrial epithelium also exhibit both message and protein of TNFα (Hunt *et al.*, 1992). Thus alternative sources of TNFα may be implicated in the processes of apoptosis. Interestingly, there is a significant increase in TNFα mRNA in the late secretory phase (Tabibzadeh *et al.*, 1994), and it has been suggested that excess production of TNFα in association with oestrogen withdrawal may be associated with the development of apoptosis in human endometrium. We are not aware of any published data concerning TNFα message or protein in 'pseudo-pregnant' endometrium, i.e. after 'luteal rescue'.

The nature and extent of apoptosis in the human endometrium at different stages of the female menstrual cycle has recently been described in detail (Tabibzadeh *et al.*, 1994). Apoptosis was found to occur at a low frequency in proliferative endometrium, then increased during the secretory phase in the glandular cells (Tabibzadeh *et al.*, 1994). No apoptosis was found in the stroma, including the lymphoid aggregates. These observations suggest that the localization of bcl-2 in the glandular component of the late secretory endometrium does not inhibit apoptosis at that stage of the cycle, although it is possible that the high intensity of bcl-2 in the glandular epithelium during the proliferative phase may reflect this function. Our observations that CD56 positive lymphocytes express *bcl-2* is in keeping with the absence of histological evidence of apoptosis in that region.

Any discussion of the significance of the immunolocalization of bcl-2 now has to be tempered by the observation that bcl-2 is only the prototype of an extended family of similar proteins which inhibit apoptosis. In particular, it is now clear that the action of *bcl-2* depends acutely on the concentration of and interaction with a potential antagonist protein, bax (Oltvai *et al.*, 1993). At present, nothing is known about the localization or level of expression of bax within human endometrium, nor are there any data about the level of expression of potential *bcl-2* effectors/antagonists such as *bcl-xL* or *bcl-xS* (Boise *et al.*, 1993). Until the nature and extent of interactions between these genes in the endometrium is clarified, the physiological significance of bcl-2 in the endometrium will remain unresolved.

In summary, we conclude from these data that *bcl-2* is expressed in the human endometrium at all stages of the menstrual cycle. We found no evidence to support a simple relationship between *bcl-2* expression and endometrial maintenance. Further research into the level of expression of bcl-2 antagonists such as bax, bcl-x-l and bcl-x-s will be required before the role of this complex gene family in the endometrium can be fully understood.

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References

Baer, R. (1994) Bcl-2 breathes life into embryogenesis. *Am. J. Pathol.*, **145,** 7–10.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G. and Thompson, C.B. (1993) *Bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. *Cell,* **74,** 597–608.

Cohen, J.J. and Duke, R.C. (1992) Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol.,* **10,** 267–293.

Djahanbakhch, O., McNeilly, A.S., Hobson, B.M. and Templeton, A.A. (1981a) A rapid luteinizing hormone radioimmunoassay for the prediction of ovulation. *Br. J. Obstet. Gynaecol.,* **88,** 1016–1020.

Djahanbakhch, O., Swanston, I.A., Corrie, J.E.T. and McNeilly, A.S. (1981b) Prediction of ovulation by progesterone. *Lancet,* **ii,** 1164–1165.

Gompel, A., Sabourin, J.C., Martin, A., Yaneva, H., Audouin, J., Decrois, Y. and Poitout, P. (1994) Bcl-2 expression in normal endometrium during the menstrual cycle. *Am. J. Pathol.,* **144,** 1195–1202.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature,* **348,** 334–336.

Hopwood, D. and Levison, D.A. (1976) Atrophy and apoptosis in the cyclical human endometrium. *J. Pathol.,* **119,** 159–165.

Hunt, J.S., Chen, H.L., Hu, X.L. and Tabibzadeh, S. (1992) Tumour-necrosis-factor-alpha messenger-ribonucleic-acid and protein in human endometrium. *Biol. Reprod.,* **47,** 141–147.

Hurwitz, A. and Adashi, E.Y. (1992) Ovarian follicular atresia as an apoptotic process: a paradigm for programmed cell death in endocrine tissues. *Mol. Cell. Endocrinol.*, **84,** C19-C23.

Illingworth, P.J., Reddi, K., Smith, K. and Baird, D.T. (1990) Pharmacologic 'rescue' of the corpus luteum results in increased inhibin production. *Clin. Endocrinol.*, **33,** 323–332.

Kelly, R.W. (1994) Pregnancy maintenance and parturition: the role of prostaglandin in manipulating the immune and inflammatory response. *Endocr. Rev.*, **15,** 684–706.

Kerr, J.F.K., Wyllie, A.H. and Currie, A.H. (1972) Apoptosis, a basic biological phenomenon—with wider implications in tissue kinetics. *Br. J. Cancer,* **26,** 239–245.

Kerr, J.F., Winterford, C.M. and Harmon, B.V. (1994) Apoptosis. Its significance in cancer and cancer therapy. *Cancer,* **73,** 2013–2026.

Kyprianou, N. and Isaacs, J.T. (1988) Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology,* **122,** 552–562.

Nawaz, S., Lynch, M.P., Galand, P. and Gerschenson, L.E. (1987) Hormonal regulation of cell death in rabbit uterine epithelium. *Am. J. Pathol.*, **127,** 51–59.

Noyes, R.W., Hertig, A.T. and Rock, J. (1950) Dating the endometrial biopsy. *Fertil. Steril.*, **1,** 33–35.

Oltvai, Z.N., Milliman, C.L. and Korsmeyer, S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell,* **74,** 609–619.

Otsuki, Y., Misaki, O., Sugimoto, O., Ito, Y., Tsujimoto, Y. and Akao, Y. (1994) Cyclic bcl-2 gene expression in human uterine endometrium during human menstrual cycle. *Lancet,* **344,** 28–29.

Tabibzadeh, S. (1994) Cytokines and the hypothalamic-pituitary-ovarian-endometrial axis. *Hum. Reprod.*, **9,** 947–967.

Tabibzadeh, S., Kong, Q.F., Satyaswaroop, P.G., Zupi, E., Marconi, D., Romanini, C. and Kapur, S. (1994) Distinct regional and menstrual cycle dependent distribution of apoptosis in human endometrium. Potential regulatory role of T-cells and TNFα. *Endocr. J.*, **2,** 87–95.

Tsujimoto, Y. and Croce, C.M. (1986) Analysis of the structure, transcripts and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc. Natl. Acad. Sci. USA,* **83,** 5214–5218.

Vaux, D., Weissman, I.L. and Kim, S.K. (1992) Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. *Science,* **258,** 1955–1957.

Wyllie, A.H., Kerr, J.F.R., Macaskill, I.A.M. and Currie, A.R. (1973) Adrenocortical cell deletion: the role of ACTH. *J. Pathol.*, **111,** 85–94.

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Expression of tissue inhibitor of metalloproteinases-1 in the human corpus luteum after luteal rescue

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Abstract

Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a specific inhibitor of a group of proteolytic enzymes known as matrix metalloproteinases. These enzymes have been widely implicated in the process of tissue remodelling.

Extensive remodelling occurs in the corpus luteum during luteolysis unless human chorionic gonadotrophin (hCG) is produced by the early conceptus. This study aimed to investigate the expression and localisation of TIMP-1 in human corpora lutea during the luteal phase of the cycle and after luteal rescue with exogenous hCG to mimic the changes of early pregnancy. Human corpora lutea from the early (n=4), mid- (n=4) and late (n=4) luteal phases and after luteal rescue by hCG (n=4) were obtained at the time of hysterectomy. Expression of TIMP-1 was investigated in these tissues by Western blotting, immunohistochemistry, Northern blotting and *in situ* hybridisation. Luteal cells of thecal origin were distinguished from those of granulosa origin by immunostaining for 17α-hydroxylase. A 30 kDa protein consistent with TIMP-1 was detected in human corpora lutea. This protein was localised to the granulosa lutein cells in all tissues examined. TIMP-1 mRNA was found in large quantities in all glands examined and this again localised to the granulosa lutein cells. The expression and localisation of TIMP-1 did not change throughout the luteal phase and was not altered by luteal rescue. The function of this uniform expression of TIMP-1 in the corpus luteum is not clear but these data suggest that the inhibition of structural luteolysis during maternal recognition of pregnancy is not mediated by regulation of TIMP-1 expression.

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Introduction

The human corpus luteum will undergo functional and structural luteolysis unless human chorionic gonadotrophin (hCG) is produced by the implanting blastocyst (Behrman *et al.* 1993). Although this process is fundamental to ovarian function the molecular mechanisms of luteolysis and luteal rescue in the human are still uncertain. During structural luteolysis, the gland rapidly changes from the most active endocrine gland in the body with a greater blood flow per unit mass than the kidney (Ford *et al.* 1982) to a small fibrous remnant.

Such a process is likely to involve extensive remodelling of the extracellular matrix which is controlled by a group of zinc-dependent enzymes, the matrix metalloproteinases (MMPs). Alterations in metalloproteinase activity have been implicated in the spread of neoplastic tissues (Naylor *et al.* 1994) as well as in remodelling of reproductive tissues, such as the endometrium at menstruation (Hampton & Salamonsen 1994) and the follicle wall at ovulation (Reich *et al.* 1985). The activity of MMPs is rigorously controlled at several levels, including synthesis as a proenzyme requiring activation and the production of specific inhibitors, tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2 and TIMP-3). TIMP-1 is of particular interest as it has recently been demonstrated that it is the major secretory product of the ovine corpus luteum (Smith *et al.* 1993) and that its expression is transiently increased during prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced luteolysis (Juengel *et al.* 1994). Other studies have reported that TIMP-1 expression increases markedly in preovulatory granulosa cells around the time of the gonadotrophin surge (Smith G W *et al.* 1994).

This study aimed to investigate the role of TIMP-1 in the human corpus luteum by studying its expression in the corpus luteum throughout the normal luteal phase and during early pregnancy simulated by the administration of hCG in logarithmically increasing doses.

Materials and Methods

Collection of tissue

Corpora lutea were enucleated at the time of hysterectomy in 16 women undergoing surgery for benign conditions. All women were healthy, aged from 32 to 45 years with

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regular menstrual cycles and had not received any form of hormonal treatment for at least 3 months prior to taking part in the study. The corpora lutea were dated on the basis of serial urinary luteinizing hormone (LH) measurements on samples collected daily prior to operation (Djahanbakhch *et al.* 1981*a*). On this basis, four corpora lutea were classified as early (LH+1 to LH+5), four as mid (LH+6 to LH+10) and four as late (LH+11 to LH+14). In addition, four women received daily i.m. injections of hCG (Profasi; Serono Laboratories, Welwyn Garden City, Herts, UK) from LH+7 in daily doubling doses, starting at 125 IU, for 5 to 8 days until surgery. This regimen has been shown to reproduce the hormonal changes of early pregnancy (Illingworth *et al.* 1990).

The whole corpus luteum was enucleated from the ovary by blunt dissection and the ovary oversewn. The tissue was immediately divided into radial blocks in order to ensure that the whole thickness of the gland was represented in any piece. Two pieces were rapidly snap frozen in liquid nitrogen and stored at −70 °C for subsequent RNA and protein extraction. A biopsy was fixed in 4% paraformaldehyde for 24 h and embedded in paraffin wax for subsequent immunohistochemistry and another biopsy was frozen in embedding medium (Tissue-Tek OCT compound; Miles Inc., Elkhart, IN, USA) and stored at −70 °C until frozen sections were cut. In each case an endometrial biopsy was also fixed in paraformaldehyde and processed into paraffin wax for luteal-phase dating by tissue morphometry (Li *et al.* 1988). Plasma was taken before surgery and progesterone concentration was measured by a standard RIA (Djahanbakhch *et al.* 1981*b*).

This study was approved by the local Reproductive Medicine Ethics Committee and informed consent was obtained from all patients prior to tissue collection.

Immunohistochemistry

All reagents used were obtained from Sigma Chemical, Poole, Dorset, UK unless indicated otherwise. Paraffin-wax sections (5 μm) were cut onto poly-L-lysine (50 μg/l)-coated slides and then dewaxed and rehydrated. As preliminary experiments indicated that microwave antigen retrieval (Shi *et al.* 1993) was necessary for the detection of TIMP-1, all sections underwent microwaving at full power (1000 W) in 0·01 M citrate buffer pH 6 for 10 min. After standing for 20 min, the sections were washed in Tris-buffered saline (TBS) pH 8 and non-specific binding was blocked by normal rabbit serum (Dako Ltd, High Wycombe, Bucks, UK) diluted 1:5 in TBS containing 5% (w/v) BSA for 20 min. Sections were incubated overnight at 4 °C with a 1:20 dilution of monoclonal mouse anti-TIMP-1 antibody (TIMP-1 (Ab-1); Cambridge Bioscience, Cambridge, Cambs, UK) in TBS. This antibody has less than 0·01% cross-reactivity with TIMP-2 protein. Antibody binding was indicated by an avidin–biotin alkaline phosphatase (Dako Ltd) reaction with a biotinylated rabbit anti-mouse (Dako Ltd) secondary antibody and substrate to give a red end product (Alkaline Phosphatase Substrate Kit I; Vector Laboratories, Peterborough, Cambs, UK). Sections were washed in water, counterstained with haematoxylin, dehydrated through graded alcohols and mounted with pertex mounting medium (Cellpath, Hemel Hempstead, Herts, UK).

As negative controls, serial sections were incubated with mouse IgG (Vector Laboratories) in place of the primary antibody at the same concentration (5 μg/ml) and in further control sections the primary antibody was omitted. Serial sections were also stained for 17α-hydroxylase using a polyclonal rabbit antibody (kindly provided by Professor M R Waterman, Vanderbilt University, Nashville, TN, USA) at a 1:750 dilution. In this case, after microwave antigen retrieval, normal goat serum (SAPU, Carluke, Strathclyde, UK) was used to block non-specific binding and specific binding was detected using biotinylated goat anti-rabbit immunoglobulins (Dako Ltd). The intensity of staining was graded by an observer blinded to the tissue identity. Staining was classified as − if absent, + if weakly present, ++ if moderate and +++ if strong.

Western blotting

Protein was extracted on ice into 0·1% SDS and protein content was estimated by the method of Bradford (1976). Protein (200 μg) was denatured by boiling in sample buffer containing β-mercaptoethanol and separated by electrophoresis on an 11% polyacrylamide gel. Proteins were electrophoretically transferred onto nitrocellulose membrane (Amersham International plc, Aylesbury, Bucks, UK). After transfer the blot was washed briefly in TBS containing 0·05% (v/v) Tween (TBST) and incubated in 10% (w/v) dried skimmed milk powder in TBST. Primary antibody was used in a concentration of 2 μg/ml in TBST and after washing in TBST the membrane was incubated with biotinylated goat anti-rabbit immunoglobulins Streptavidin alkaline phosphatase (Dako Ltd) was added after washing in TBST and after further washing visualisation was achieved using a red chromogen (Alkaline Phosphatase Substrate Kit I; Vector Laboratories). Molecular weight markers (Biorad Laboratories, Hemel Hempstead, Herts, UK) were run in an adjacent lane to calculate the weight of the detected proteins. Ovine luteal cell-conditioned medium (kindly provided by Professor M F Smith, University of Missouri, Columbia, MO, USA) was used as a positive control for TIMP-1. The intensity of each band was determined by computer aided densitometric image analysis (NIH Image 1·55; NIH, MD, USA).

Northern blotting

Total RNA was isolated by the method of Chomczynski & Sacchi (1987) using a commercial kit (RNAzolB

Biogenesis, Bournemouth, Dorset, UK) and its concentration was determined by absorption at 260 nm. Total RNA (30 μg) was denatured, electrophoresed in a 1·5% formaldehyde-agarose gel and transferred to a nylon membrane (Amersham International plc) by capillary action in 20 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate) at pH 7. The RNA was fixed onto the membranes by u.v. cross-linkage (Spectronics Corporation, New York, NY, USA). Membranes were prehybridised for 5 h in 15 ml hybridisation buffer (0·5 M sodium phosphate, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS, 6·7% (v/v) deionised formamide) at 65 °C. A human TIMP-1 cDNA probe (kindly provided by Dr E Bone, British Biotech Pharmaceuticals Ltd, Oxford, Oxon, UK) was labelled with 50 μCi ^{32}P dCTP by the random priming method using a commercial kit (Amersham International plc) and added to the hybridisation buffer. Hybridisation was performed at 65 °C for 20 h. The membranes were washed twice at 65 °C with 2 × SSC for 15 min and once more with 2 × SSC/0·1% SDS at 65 °C for 15 min. The blots were laid down to a phosphor screen for 24 h and developed and quantified using a phosphorimager computer (Molecular Dynamics, Sevenoaks, Kent, UK). To correct for minor differences in RNA loading the blots were stripped in stripping buffer (5 mM Tris pH 8·0, 0·3 mM EDTA, 0·1 × Denhardt's reagent) for 2 h at 65 °C and reprobed in the same manner with a cDNA probe to human β-actin (Clontech, Palo Alto, CA, USA). We found no differences in the level of β-actin expression during the different stages of the luteal phase. The molecular size of the transcripts was determined by running commercial RNA markers (Promega, Southampton, Hants, UK) in an adjacent lane. The ratio of relative intensities for TIMP-1 to β-actin after normalisation by logarithmic transformation was used for data analysis.

In situ *hybridisation*

In situ hybridisation was performed on frozen sections using an antisense TIMP-1 ^{35}S-labelled riboprobe. A TIMP-1 cDNA construct in the transcription vector pGEM 4-Z was obtained from Dr E Bone. Antisense and sense riboprobes incorporating ^{35}S-labelled UTP (Amersham International plc) were synthesised using a commercial kit (Promega). The antisense probe was generated from the vector linearised by BamHI (Promega) using T7 RNA polymerase. The sense probe which was used as the negative control was generated from the vector linearised by EcoRI (Promega) using SP6 RNA polymerase.

Frozen sections (6 μm) on poly-L-lysine-coated slides were quickly thawed and fixed in 4% paraformaldehyde for 5 min at room temperature. After washing in 0·1 M sodium phosphate, slides were transferred into 0·1 M triethanolamine (TEA) and acetylated in 0·25% (v/v) acetic anhydride (BDH Laboratory Supplies, Poole, Dorset, UK) in TEA for 10 min. The sections were washed in 2 × SSC before dehydration and delipation through graded alcohols and chloroform. Slides were air dried and incubated with prehybridisation buffer (50% formamide, 10 mM dithiothreitol (DTT), 1 × Denhardt's, 4 × STE (1 × STE is 0·1 M NaCl, 10 mM Tris–HCl pH 8, 1 mM EDTA), 125 μg yeast tRNA/ml, 125 μg salmon sperm DNA/ml) for 1 h at 45 °C. Excess prehybridisation buffer was removed and slides were incubated with 1×10^6 c.p.m. radiolabelled probe in 40 μl hybridisation buffer (prehybridisation buffer containing 10% (v/v) dextran sulphate), sealed under Gel Bond film (ICN Biomedical Ltd, High Wycombe, Bucks, UK) and incubated overnight at 45 °C in a moist chamber. Sections were washed in 2 × SSC/2 mM DTT prior to RNase A treatment (30 mg/ml in RNase buffer (10 mM Tris, 1 mM EDTA, 0·5 M NaCl, pH 8)) for 30 min at 37 °C. The sections were sequentially washed in RNase buffer/1 mM DTT and 2 × SSC/1 mM DTT at room temperature before washing in 0·1 × SSC/1 mM DTT at 45 °C in a shaking water bath. Sections were rinsed in 0·1 × SSC then dehydrated through graded alcohols containing 0·3 M ammonium acetate and air-dried before dipping in Kodak NTB-2 photographic emulsion (IBI Ltd, Cambridge, Cambs, UK) in the dark. The emulsion was exposed in light-tight boxes containing silica gel at 4 °C for 21 days then developed (Kodak D-19) and fixed (Kodak Unifix) at 15 °C in the dark. After rinsing in running tap water, sections were counterstained with haematoxylin, dehydrated through graded alcohols into xylene and mounted in pertex mounting medium. The distribution of grains was analysed by dark-field microscopy, after image capture, using a computer-based image analysis system (Cue-2, Olympus Optical Co. UK Ltd, London, UK). The cellular composition of each field was identified by light-field microscopy.

Results

Plasma progesterone concentrations

Classification of the corpora lutea by serial urinary LH measurement agreed with luteal-phase dating of endometrial biopsies using the method of Li *et al.* (1988) in all cases. Plasma progesterone concentrations were 36·36 ± 9·28 nmol/l for the early luteal tissue, 40·35 ± 9·88 nmol/l for the mid-luteal samples and 18·80 ± 12·81 nmol/l for the late luteal samples. After luteal rescue by exogenous hCG the plasma progesterone concentrations had increased to 52·75 ± 1·09 nmol/l.

Immunohistochemistry

Immunostaining for TIMP-1 was present in corpora lutea of all stages of the luteal cycle and after luteal rescue. As

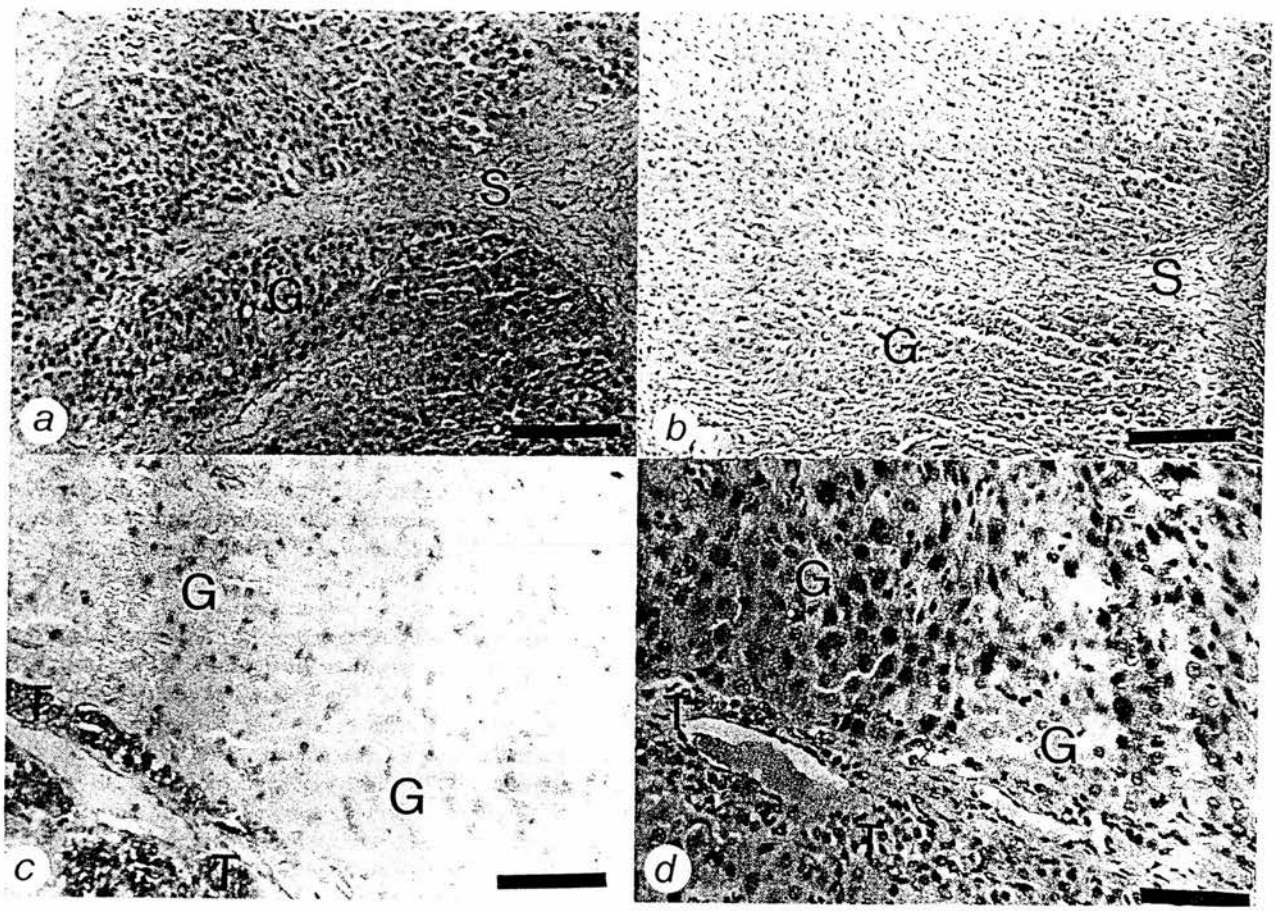

Figure 1 Immunostaining of human corpora lutea: (*a*) TIMP-1 protein in the early corpus luteum (red), staining is present in the steroidogenic cells (G) and absent from the surrounding stroma (S) (scale bar=100 μm); (*b*) negative control serial section of (*a*) showing no staining of the steroidogenic cells (G) and the stroma (S) (scale bar=100 μm); (*c*) immunostaining for 17α-hydroxylase enzyme in a late luteal corpus luteum (red), the theca lutein cells are seen in discrete clumps (T) at the periphery of the granulosa lutein cells (G) (scale bar=50 μm); (*d*) serial section of (*c*) stained for TIMP-1, the theca lutein cells (T) are relatively free from staining and the granulosa lutein cells (G) show specific TIMP-1 immunostaining (red) (scale bar=50 μm).

can be seen in Fig. 1*a*, staining was localised to the steroidogenic cells of the gland and absent from the negative controls (Fig. 1*b*). There were no differences in the localisation of immunostaining for TIMP-1 during the luteal cycle or after luteal rescue. In particular, the regressing corpus luteum of the late luteal phase showed no local areas of altered TIMP-1 immunostaining within the tissue section. The intensity of staining in all sections was classified as ++ or +++ with no obvious pattern of variation throughout the luteal cycle or after luteal rescue. The steroidogenic cells derived from the theca were identified by immunostaining serial sections for the enzyme 17α-hydroxylase. Figure 1*c* shows the theca lutein cells to be present in discrete clumps along the periphery of the granulosa lutein cells. This pattern of staining was similar to that reported by Tamura *et al.* (1992). The theca cells were relatively free from immunostaining for TIMP-1 as can be seen in Fig. 1*d*.

Western blotting

Western blotting showed a single band at 30 kDa as shown in Fig. 2. This is consistent with the molecular weight of TIMP-1 which has previously been reported (Smith G V *et al.* 1994) and the same band could be detected in ovine luteal cell-conditioned medium known to contain TIMP-1. This protein was detected in protein samples from human luteal tissue from all stages of the cycle. The intensity of the detected protein band in luteal cell extracts was similar at all stages of the luteal cycle and after luteal rescue. The width of the detected band suggested there may be a heterologous population of TIMP-1 protein

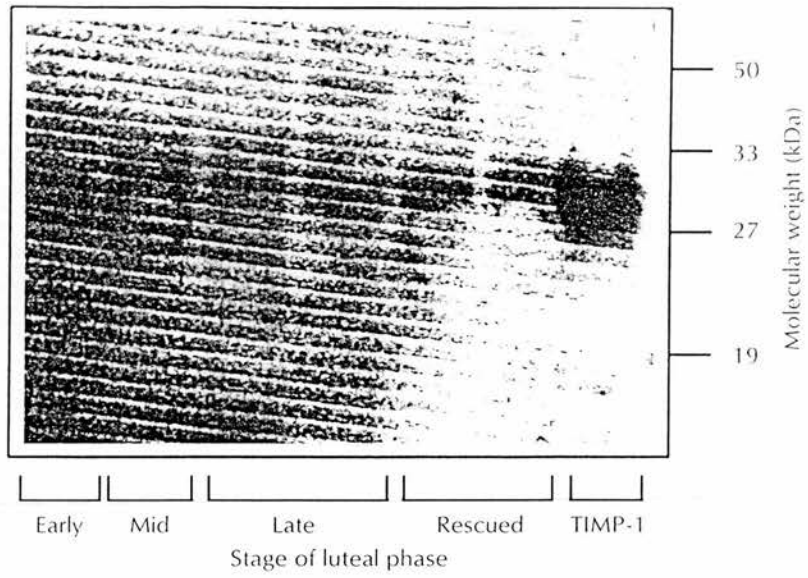

Figure 2 Western blot for TIMP-1 protein in luteal tissue homogenates from different stages of the luteal phase and after luteal rescue by exogenous hCG. Ovine luteal cell-conditioned medium known to contain large quantities of TIMP-1 was used as a positive control. The migration of molecular weight markers is indicated at the right of the figure.

with different glycosylation patterns. We found no consistent differences in protein band width during the different stages of the luteal phase however.

Northern blotting

A single band approximately 0·9 kb in length was detected in total RNA extracted from human corpora lutea by Northern blotting. This size was consistent with TIMP-1 message reported in humans (Rapp *et al.* 1990) and sheep (Smith G W *et al.* 1994). As shown in Fig. 3, TIMP-1 mRNA was abundant and could be found at all stages of the luteal cycle and after luteal rescue. Densitometric quantification of the intensity of the message standardised for tissue β-actin expression demonstrated no differences of level of TIMP-1 message during the luteal cycle and after luteal rescue (Fig. 4).

In situ hybridisation

As shown in Fig. 5*a*, message for TIMP-1 was uniformly distributed throughout the granulosa lutein cells of the corpus luteum. This distribution was only seen in the sections incubated with the antisense probe and was absent from control sections incubated with the sense probe (Fig. 5*b*). The localisation of TIMP-1 mRNA in luteal cells was consistent with the localisation of TIMP-1 protein (Fig. 1*a*). Luteal cells of thecal origin had much lower grain concentrations than those of granulosa cell origin (Fig. 5*c* and *d*). The localisation of TIMP-1 mRNA did not change throughout the luteal cycle or after luteal rescue.

Discussion

This study is the first to demonstrate the expression and localisation of TIMP-1 in the human corpus luteum. TIMP-1 mRNA has previously been demonstrated in the corpus luteum of a number of other species including the sheep (Smith G W *et al.* 1994), the cow (Juengel *et al.* 1994) and the pig (Smith M F *et al.* 1994). Indeed it has previously been shown that TIMP-1 is one of the major products of the ovine corpus luteum and our data suggest that TIMP-1 is similarly abundant in the luteal cells of the human ovary. The immunohistochemistry and immunoblotting demonstrate specific binding to a protein of the appropriate molecular weight while the Northern analysis and *in situ* hybridisation experiments confirmed the source of specific mRNA expression.

The TIMP-1 protein and mRNA were found to be present specifically in the granulosa–lutein cell types of the corpus luteum. Human preovulatory granulosa cells have previously been shown to produce metalloproteinase inhibitor activity *in vitro* (Morgan *et al.* 1994) and contain abundant TIMP-1 mRNA (Rapp *et al.* 1990). TIMP-1 is expressed in the granulosa cells of the preovulatory follicle in the sheep at a 15-fold higher level than in the theca cells of the same follicle (Smith G W *et al.* 1994). We identified

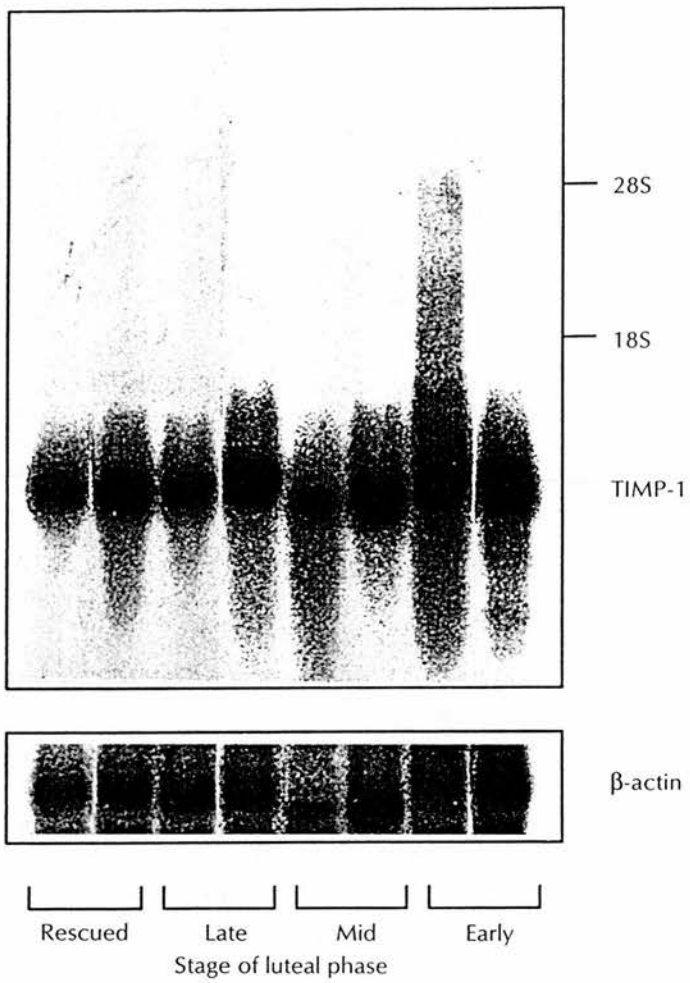

Figure 3 Northern blot of TIMP-1 mRNA using total RNA extracted from corpora lutea at different stages of the luteal phase and after luteal rescue by exogenous hCG. The positions of the ribosomal 28S and 18S bands are indicated. The control bands detected after stripping the blot and re-probing for β-actin are indicated below the TIMP-1 blot.

the steroidogenic cells of thecal origin in the corpus luteum by immunostaining with an antibody to 17α-hydroxylase. This enzyme is not present in the granulosa lutein cells and has been used to identify cells of thecal origin in human corpora lutea (Tamura *et al.* 1992, Rodger *et al.* 1995). We found that there was little if any immunostaining or *in situ* hybridisation for TIMP-1 in the theca lutein cells of the human corpus luteum. As the localisation of TIMP-1 appeared to be confined to the large steroidogenic cells of the corpus luteum it is unlikely that non-steroidogenic cells present in the corpus luteum, such as macrophages and T-lymphocytes (Brannstrom *et al.* 1994), contribute to TIMP-1 production but this remains to be established.

No change was found in either the site or the intensity of expression of TIMP-1 during the luteal phase or following luteal rescue with hCG. This was surprising, as luteal expression of TIMP-1 has previously been show rise for up to 8 h during $PGF_{2\alpha}$-induced luteolysis i cow (Juengel *et al.* 1994). The same group loca TIMP-1 mRNA by *in situ* hybridisation during lute and found that scattered individual cells within the oidogenic tissue expressed much higher levels than surrounding cells (Smith G W *et al.* 1994). This patte expression was unlike the uniform grain distribution within ovine follicular granulosa cells (Smith G W 1994). In the human corpus luteum we found expression of TIMP-1 is uniform in the granulosa l cell population and this distribution is maintained thro out the luteal cycle. The different patterns of expr are probably due to a more gradual luteolytic proc women. Although we studied four corpora lutea fro late luteal phase, all the corpora lutea studied wer

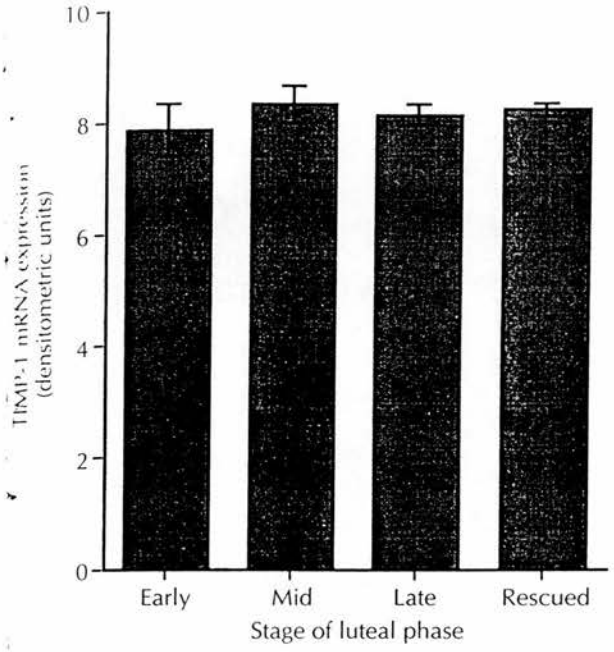

Figure 4 TIMP-1 mRNA in corpora lutea from different stages of the luteal phase and after luteal rescue by exogenous hCG. Message for TIMP-1 detected by Northern blotting was quantified by computer-aided densitometry corrected for minor loading variations by comparison of β-actin levels. Values are means ± S.E.M., $n=4$.

functional, in that progesterone secretion was continuing. Unlike the ruminant, luteolysis in women does not result from a surge of uterine prostaglandin and is therefore likely to be a more gradual process (Auletta & Flint 1988). In contrast, in cattle, prostaglandin treatment produces co-ordinated luteolysis which allows changes induced in a proportion of cells, such as apoptosis, to be detected more easily (Juengel *et al.* 1993). The more gradual fall in luteal function in women may mask the increased TIMP-1 expression seen within individual cells during luteolysis in the ovine gland.

Luteal involution is inhibited during maternal recognition of pregnancy by hCG acting through the LH receptor (Behrman *et al.* 1993). Expression of TIMP-1 in preovulatory granulosa cells increases during luteal formation at the time of the gonadotrophin surge (Smith G W *et al.* 1994). At this stage the granulosa cells contain LH receptors (Richards & Midgley 1976) and it is possible that the increase in TIMP-1 is partially mediated through stimulation of this receptor. In the rat, LH stimulates secretion of a TIMP-like protein in granulosa cell cultures and TIMP-1 mRNA is increased by LH (Mann *et al.* 1991, Morgan *et al.* 1994). However, it does not appear that further stimulation of the corpus luteum by hCG, as occurs in normal human pregnancy, provokes any further increase in TIMP-1 synthesis. This may be a species-related difference, as Morgan *et al.* (1994) were unable to show any increase in TIMP-1 in response to LH in cultured human granulosa cells. TIMP-1 expression in women may be controlled by other hormones, such as progesterone. Progesterone receptors have been identified in the human corpus luteum (Suzuki *et al.* 1994) and the progesterone antagonist RU486 can reduce TIMP activity in granulosa cell cultures (Morgan *et al.* 1994).

The significance of the TIMP-1 produced by the human corpus luteum remains unclear but its production in large quantities throughout the luteal phase suggests an important role. In view of this, Juengel *et al.* (1994) have suggested that TIMP-1 expression reflects the production of endogenous metalloproteinases and acts as a protective mechanism to limit specific proteinase action. However, as described in this paper, TIMP-1 mRNA is abundant in the corpus luteum, it is one of the most abundant mRNAs in luteinised granulosa cells (Rapp *et al.* 1990) and TIMP-1 is the major protein product of the ovine corpus luteum (Smith *et al.* 1993). In contrast, preliminary results (Duncan *et al.* 1995) suggest that the mRNAs for MMP-2 and MMP-9 are present in far lower concentrations than that of TIMP-1. As TIMP-1 binds to and inactivates MMPs in a 1:1 ratio (Matrisian 1990) it is probable that, in the corpus luteum, MMPs are functioning in an environment containing a high level of specific inhibitor. For MMPs to function in this environment it is likely that the interaction between MMPs and TIMPs is at a local cellular rather than a tissue-wide level. It may be that local tissue remodelling is regulated by the expression of proMMPs and their subsequent activation to active MMPs, rather than control of inhibition. It is consequently difficult to assess the function of TIMP-1 in isolation, when the activity and production of MMPs and other inhibitors such as TIMP-2 remain unclear during the luteal cycle, particularly as it has recently been reported that TIMP-2 is also produced by the ovine corpus luteum (Smith *et al.* 1995).

TIMP-1 has other properties which may contribute to a critical role in maintenance of the corpus luteum as well as the inhibition of metalloproteinase activity. TIMP-1 promotes the proliferation of fibroblasts and endothelial cells and may be a paracrine or autocrine growth factor (Hayakawa *et al.* 1992). This may be important to facilitate the high levels of angiogenesis during corpus luteum formation (Reynolds *et al.* 1992). It is also possible that TIMP-1 has a role in cell migration (Smith G W *et al.* 1994) or protection against neoplastic stimuli during periods of tissue growth and neovascularisation. A recent publication (Boujrad *et al.* 1995) has raised the possibility that TIMP-1, particularly in combination with procathepsin-L, may be involved in regulating steroidogenesis. Boujrad *et al.* (1995) found that a locally produced follicle-stimulating hormone-responsive factor which stimulates both Leydig and granulosa cell steroidogenesis is the TIMP-1–procathepsin-L complex. Both TIMP-1 and this complex were found to stimulate steroidogenesis in a

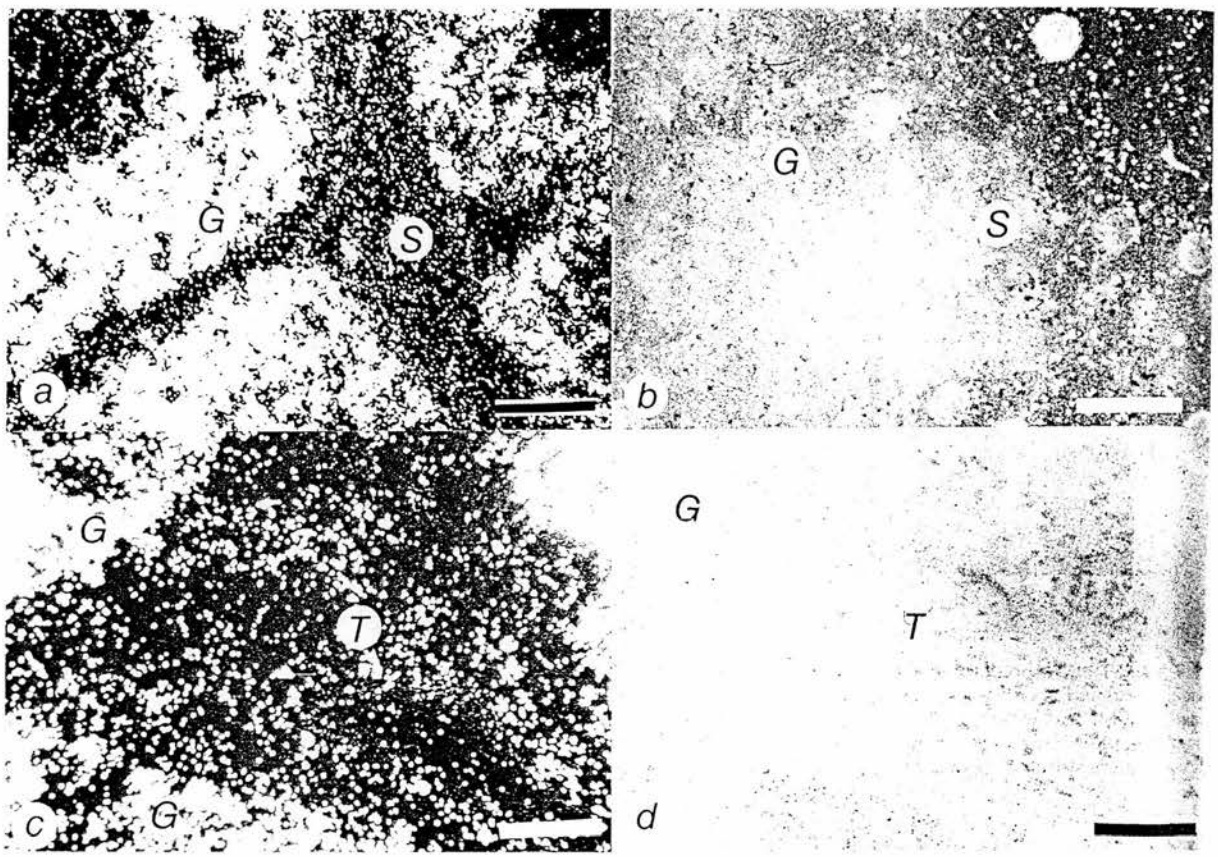

Figure 5 *In situ* hybridisation of human corpora lutea for TIMP-1 mRNA: (*a*) dark field of TIMP-1 mRNA in the early corpus luteum, grains are found in the steroidogenic cells (G) at much higher levels than the surrounding stroma (S) (scale bar=100 μm); (*b*) dark-fiel negative control serial section of (*a*) using sense TIMP-1, there are no differences in grain localisation in the steroidogenic cells (G) an the stroma (S) (scale bar=100 μm); (*c*) dark field of luteal TIMP-1 mRNA showing uneven distribution of grains within the steroidoger cell layer (scale bar=50 μm); (*d*) light field of (*c*) showing the granulosa lutein cells (G) and the theca lutein cells (T), grains are preser at much higher levels in the granulosa lutein cells (G) than the theca lutein cells (T) (scale bar=50 μm).

cyclic AMP-independent manner with a bioactivity similar to saturating amounts of hCG. Although the effects of TIMP-1 and the procathepsin-L complex on luteal steroidogenesis are not yet known, it is notable that the corpus luteum has a very high level of synthesis of both TIMP-1 and steroids.

In summary, TIMP-1 is an abundant secretory product of the human corpus luteum during both the normal menstrual cycle and in simulated early pregnancy. The physiological role of the TIMP-1 is unclear but may involve the facilitation of steroidogenesis and the protection of the ovary against the effects of local metalloproteinase production.

Acknowledgements

The authors would like to express grateful thanks to Dr E Bone at British Biotech Pharmaceuticals Ltd for providing the TIMP-1 cDNA probe and to Professor M Waterman at the University of Kentucky for providing antibody to 17α-hydroxylase. Professor M F Smith of University of Missouri provided useful help and discus as did G M Cowen, M R Millar, S F Lunn and H Fraser. Mr E Pinner and Mr T McFetters exp prepared the figures. W C D is supported by a clin research fellowship from the Wellcome Trust.

References

Auletta FJ & Flint APF 1988 Mechanisms controlling corpus lutei function in sheep, cows, nonhuman primates, and women espe in relation to the time of luteolysis. *Endocrine Reviews* **9** 88–105

Behrman HR, Endo T, Aten RF & Musicki B 1993 Corpus lute function and regression. *Reproductive Medicine Review* **2** 153–18

Boujrad N, Ogwuegbu SO, Garnier M, Lee C-H, Martin BM & Papadopoulos V 1995 Identification of a stimulator of steroid hormone synthesis isolated from testis. *Science* **268** 1609–1612.

Bradford MM 1976 A rapid and sensitive method for the

quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.

Brannstrom M, Pascoe V, Norman RJ & McClure N 1994 Localization of leukocyte subsets in the follicle wall and in the corpus-luteum throughout the human menstrual-cycle. *Fertility and Sterility* **61** 488–495.

Chomczynski P & Sacchi N 1987 Single step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.

Djahanbakhch O, McNeilly AS, Hobson BM & Templeton AA 1981*a* A rapid luteinizing hormone radioimmunoassay for the prediction of ovulation. *British Journal of Obstetrics and Gynaecology* **88** 1016–1020.

Djahanbakhch O, Swanston IA, Corrie JET & McNeilly AS 1981*b* Prediction of ovulation by progesterone. *Lancet* **ii** 1164–1165.

Duncan WC, McNeilly AS & Illingworth PJ 1995 The effect of luteal rescue on the expression of matrix metalloproteinases and their inhibitors in the human corpus luteum. *Biology of Reproduction* **52** (Suppl 1) 222.

Ford SP, Reynolds LP & Magness RR 1982 Blood flow to the uterine and ovarian vascular beds of gilts during the estrous cycle or early pregnancy. *Biology of Reproduction* **27** 878–885.

Hampton AL & Salamonsen LA 1994 Expression of messenger ribonucleic acid encoding matrix metalloproteinases and their tissue inhibitors is related to menstruation. *Journal of Endocrinology* **141** R1–R3.

Hayakawa T, Yamashita K, Tanzawa K, Uchijima E & Iwata K 1992 Growth promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. *FEBS Letters* **298** 29–32.

Illingworth PJ, Reddi K, Smith K & Baird DT 1990 Pharmacologic 'rescue' of the corpus luteum results in increased inhibin production. *Clinical Endocrinology* **33** 323–332.

Juengel JL, Garverick HA, Johnson AL, Youngquist RS & Smith MF 1993 Apoptosis during luteal regression in cattle. *Endocrinology* **132** 249–254.

Juengel JL, Smith GW, Smith MF, Youngquist RS & Garverick HA 1994 Pattern of protein production by bovine corpora lutea during luteolysis and characterization of expression of two major secretory products of the regressing corpora lutea. *Journal of Reproduction and Fertility* **100** 515–520.

Li TC, Rogers AW, Dockery P, Lenton EA & Cooke ID 1988 A new method of histologic dating of human endometrium in the luteal phase. *Fertility and Sterility* **50** 52–60.

Mann JS, Kindy MS, Edwards DR & Curry TE Jr 1991 Hormonal regulation of matrix metalloproteinase inhibitors in rat granulosa cells and ovaries. *Endocrinology* **128** 1825–1832.

Matrisian LM 1990 Metalloproteinases and their inhibitors in matrix remodelling. *Trends in Genetics* **6** 121–125.

Morgan A, Keeble SC, London SN, Mase KN & Curry TE Jr 1994 Antiprogesterone (RU 486) effects on metalloproteinase inhibitor activity in human and rat granulosa cells. *Fertility and Sterility* **61** 949–955.

Naylor MS, Stamp GW, Davies BD & Balkwill FR 1994 Expression and activity of MMPs and their regulators in ovarian cancer. *International Journal of Cancer* **58** 50–56.

Rapp G, Freudstein J, Klaudiny J, Mucha J, Wempe F, Zimmer M & Scheit KH 1990 Characterisation of three abundant mRNAs from human ovarian granulosa cells. *DNA and Cell Biology* **9** 479–485.

Reich R, Tsafriri A & Mechanic GL 1985 Involvement of collagenolysis in ovulation in the rat. *Endocrinology* **116** 522–527.

Reynolds LP, Killilea SD & Redmer DA 1992 Angiogenesis in the female reproductive system. *FASEB Journal* **6** 886–892.

Richards JS & Midgley AR Jr 1976 Protein hormone action: A key to understanding ovarian follicular and luteal cell development. *Biology of Reproduction* **14** 82–94.

Rodger FE, Fraser HM, Duncan WC & Illingworth PJ 1995 Immunolocalisation of bcl-2 in the human corpus luteum. *Human Reproduction* **10** 1566–1570.

Shi S, Chaiwun B, Young I, Cote RJ & Taylor CR 1993 Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *Journal of Histochemistry and Cytochemistry* **41** 1599–1604.

Smith GW, Moor RM & Smith MF 1993 Identification of a 30 000 M_r polypeptide secreted by cultured ovine granulosa cells and luteal tissue as a tissue inhibitor of metalloproteinases. *Biology of Reproduction* **48** 125–132.

Smith GW, Goetz TL, Anthony RV & Smith MF 1994 Molecular cloning of an ovine ovarian tissue inhibitor of metalloproteinases: Ontogeny of messenger ribonucleic acid expression and *in situ* localization within preovulatory follicles and luteal tissue. *Endocrinology* **134** 344–352.

Smith GW, McCrone S, Petersen SL & Smith MF 1995 Expression of messenger ribonucleic acid encoding tissue inhibitor of metalloproteinases-2 within ovine follicles and corpora lutea. *Endocrinology* **136** 570–576.

Smith MF, Kemper CN, Smith GW, Goetz TL & Jarrell VL 1994 Production of tissue inhibitor of metalloproteinases-1 by porcine follicular and luteal cells. *Journal of Animal Science* **72** 1004–1012.

Suzuki T, Sasano H, Kimura N, Tamura M, Fukaya T, Yajima A & Nagura H 1994 Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: relationship to expression of steroidogenic enzymes. *Human Reproduction* **9** 1589–1595.

Tamura T, Kitawaki J, Yamamoto T, Osawa Y, Kominami S, Takemori S & Okada H 1992 Immunohistochemical localization of 17α-hydroxylase/C17–20 lyase and aromatase cytochrome P-450 in the human ovary during the menstrual cycle. *Journal of Endocrinology* **135** 589–595.

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Expression of tissue inhibitor of metalloproteinases-1 in the primate ovary during induced luteal regression

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Abstract

Although tissue inhibitor of metalloproteinases-1 (TIMP-1) is one of the major secretory products of the corpus luteum, the functional significance of this is not clear. In addition to its role as a specific inhibitor of the matrix metalloproteinase enzymes involved in tissue remodelling, it has recently been suggested that TIMP-1 is also a potent stimulator of steroidogenesis *in vitro*. However, in the ruminant, TIMP-1 expression increases during luteal regression. This study sought to determine (i) the effect of induced luteal regression on ovarian TIMP-1 expression in the primate and (ii) the expression of TIMP-1 in other steroidogenic and non-steroidogenic tissues.

Marmoset ovaries were studied on day 10 of the normal luteal phase and 12 and 24 h after induced luteolysis, with either gonadotrophin-releasing hormone (GnRH) antagonist or prostaglandin $F_{2\alpha}$ analogue. Ovaries from different stages of the normal ovarian cycle were also studied. Expression of TIMP-1 was investigated by isotopic *in situ* hybridisation. TIMP-1 expression was also examined in a wide range of other marmoset tissues by Northern blotting and *in situ* hybridisation. TIMP-1 was found to be highly expressed in the marmoset corpus luteum. Luteolysis induced with either prostaglandin $F_{2\alpha}$ or GnRH antagonist was associated with a significant fall in TIMP-1 expression in luteal tissue. TIMP-1 mRNA was also localised to ovarian follicles throughout the ovarian cycle. Expression occurred in the thecal layer of smaller follicles (<1·5 mm) and the granulosal layer of larger pre-ovulatory follicles. In atretic follicles, TIMP-1 was highly expressed at the interface between the thecal and granulosal cells. TIMP-1 was found to be predominantly expressed in steroidogenic tissues, particularly the ovary, adrenal and placenta.

These data support a role for changes in TIMP-1 expression in tissue remodelling in the ovary and are consistent with an additional function of TIMP-1 as a facilitator of steroidogenesis.

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Introduction

Unless chorionic gonadotrophin (CG) is produced by the implanting blastocyst, the primate corpus luteum will stop secreting progesterone and become a small fibrous remnant. The mechanisms of functional and structural luteolysis in the primate are still poorly understood (Behrman *et al.* 1993). The highly vascular corpus luteum is formed from the dominant follicle and in turn becomes the avascular corpus albicans in a matter of weeks. This process involves extensive tissue remodelling (Luck & Zhao 1995). The matrix metalloproteinases are a group of zinc-dependent proteolytic enzymes which have been implicated in remodelling of the extracellular matrix (Birkedal-Hansen 1995). The activity of these enzymes is controlled at several levels, including their synthesis as pro-enzymes, enzyme activation and the production of specific inhibitors (Matrisian 1990). The corpus luteum is known to produce specific tissue inhibitors of metalloproteinases (Smith *et al.* 1993, 1995). One of these, tissue inhibitor of metalloproteinases-1 (TIMP-1), is of particular interest as it is one of the major products of the corpus luteum of many species, including the sheep (Smith *et al.* 1993), cow (Juengel *et al.* 1994), pig (Smith *et al.* 1994*b*) and the human (Duncan *et al.* 1996).

The role of TIMP-1 in the corpus luteum has yet to be elucidated. Juengel *et al.* (1994) found that the expression of TIMP-1 increased after prostaglandin $F_{2\alpha}$-induced luteolysis in the cow and postulated that it may have an important role in tissue remodelling during luteolysis. However, it is not known whether TIMP-1 expression is increased during luteolysis in the primate. We did not observe any change in TIMP-1 expression over the functional lifespan of the human corpus luteum (Duncan *et al.* 1996) implying that TIMP-1 may have other functions in the primate corpus luteum. Boujrad *et al.* (1995)

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found that a locally produced follicle-stimulating hormone (FSH)-responsive factor, which stimulated both Leydig and granulosal cell steroidogenesis, was the TIMP-1–procathepsin-L complex. Both TIMP-1 and this complex were found to stimulate steroidogenesis in a cAMP-independent manner with a bioactivity similar to saturating amounts of human CG. It is therefore possible that one role of TIMP-1 is to facilitate steroidogenesis.

This study aimed to investigate the expression of TIMP-1 during luteolysis in the primate corpus luteum. The marmoset monkey was used as a model, as luteolysis can be induced by both prostaglandin $F_{2\alpha}$ and luteinising hormone (LH) withdrawal (Fraser *et al.* 1995*a*). As whole primate ovaries were studied, we were also able to describe the localisation of TIMP-1 mRNA in ovarian follicles. In order to investigate the possibility of a general role for TIMP-1 in steroidogenesis, TIMP-1 expression was studied in a wide range of endocrine and non-endocrine tissues.

Materials and Methods

Collection of tissue

Captive-bred common marmoset monkeys (*Callithrix jacchus jacchus*) were maintained in a colony which has been closed since 1973. All experiments were carried out in accordance with the *Animals (Scientific Procedures) Act* 1986. To confirm normal ovulatory cycles, plasma samples were collected by femoral venepuncture on alternate days and stored at − 20 °C until required. These samples were assayed for progesterone to determine the date of ovulation and the luteal phase duration (Smith *et al.* 1990). The marmoset normally ovulates two or three follicles and has a functional luteal phase of approximately 18 days.

Ovaries were collected on day 10 of the luteal phase, as previously described (Fraser *et al.* 1995*a*), from untreated control animals (n=4) and animals treated with: the prostaglandin $F_{2\alpha}$ analogue, cloprostenol (1 μg i.m. injection; Planate (Coopers Animal Health Ltd, Crewe, Cheshire, UK)) at 12 h (n=3) and 24 h (n=3) previously; the gonadotrophin-releasing hormone (GnRH) antagonist, antarelix ([*N*-Ac-D-Nal^1,D-pCl-Phe^2,D-Pal^3,D-(Hic)^6, Lys(iPr)^8,D-Ala^{10}]-GnRH (Deghenghi *et al.* 1993) (500 μg/kg s.c. injection; Europeptides (GEIE), Argenteuil, Val-D'Oise, France) 12 h (n=3) and 24 h (n=3) previously. Whole ovaries were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin wax. In addition, some marmoset ovaries were available which had been frozen in embedding medium (Tissue-Tek OCT compound; Miles Inc., Elkhart, IN, USA) after removal. These included follicular phase ovaries (n=4), luteal phase ovaries (n=3) and other ovaries where luteolysis had been induced as described above (n=8). Frozen sections were prepared from these ovaries and stored at − 70 °C until use.

A bank of normal marmoset tissues, collected from this and other experiments, was also utilised. These tissues were removed immediately *post-mortem*. A piece of each tissue was snap-frozen in liquid nitrogen and stored at − 70 °C for subsequent RNA extraction. Another piece was frozen in embedding medium, and stored at − 70 °C until frozen sections were prepared. All reagents used were obtained from Sigma Chemical Co., Poole, Dorset, UK unless otherwise indicated.

In situ *hybridisation*

Isotopic *in situ* hybridisation was performed on both fixed and frozen sections using ^{35}S-labelled riboprobes. A full-length human TIMP-1 cDNA construct in the transcription vector pGEM 4-Z was kindly supplied by British Biotech Pharmaceuticals Ltd, Oxford, Oxon, UK. Antisense and sense riboprobes, incorporating ^{35}S-labelled UTP (Amersham International plc, Aylesbury, Bucks, UK), were synthesised using a commercial kit (Promega, Southampton, Hants, UK). The antisense probe was generated from the plasmid vector linearised by KpnI (Promega) using T7 RNA polymerase (Promega). The sense probe was used as the negative control. This was generated from the plasmid vector linearised by HindIII (Promega) using SP6 RNA polymerase. Sections of mid-luteal human corpus luteum (Duncan *et al.* 1996) were used as a positive control in each experiment.

Fixed sections (5 μm) on poly-L-lysine (50 mg/l)-coated slides were dewaxed in fresh xylene, and rehydrated through graded alcohols. The slides were then incubated with proteinase K (5 μg/ml) in buffer (100 mM Tris, 50 mM EDTA, pH 8) for 30 min at 37 °C. Where frozen sections were used, 5 μm sections on poly-L-lysine-coated slides were quickly thawed, fixed in 4% paraformaldehyde for 5 min, at room temperature, and washed in 0·1 M sodium phosphate. Both types of slide were then treated identically. They were rinsed first in water and then in 0·1 M triethanolamine (TEA), pH 8.

After the rinsing, the slides were acetylated in 0·25% (v/v) acetic anhydride (BDH Laboratory Supplies, Poole, Dorset, UK) in TEA. They were then washed in 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate), pH 7, and dehydrated through graded alcohols before being dried under vacuum in a desiccator for 1 h at room temperature. Then 100 μl hybridisation buffer (50% deionised formamide, 10% dextran sulphate, 1 × Denhardt's solution (0·02% Ficoll 400/0·02% polyvinylpyrrolidone/0·02% BSA), 0·5 mg/ml yeast tRNA, 10 mM dithiothreitol (DTT), 0·3 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8) containing 1×10^6 c.p.m. radiolabelled probe were added to each section. The slides were covered with a hydrophobic coverslip (Gel Bond; ICN Biomedical Ltd, High Wycombe, Bucks, UK) and incubated overnight at 55 °C in a moist chamber.

The following day the coverslips were washed off in 4 × SSC. After several rinses in 4 × SSC, the slides were treated with RNase A (20 μg/ml) in RNase buffer (10 mM Tris, 1 mM EDTA, 0·5 M NaCl, pH 8) for 30 min at 37 °C. The sections were desalted by rinsing in 2 × SSC/1 mM DTT, followed by 1 × SSC/1 mM DTT and 0·5 × SSC/1 mM DTT at room temperature. The slides were then washed for 30 min in 0·1 × SSC at 70 °C in a shaking water bath. After being rinsed in 0·1 × SSC/1 mM DTT at room temperature, the sections were dehydrated through graded alcohols containing 1 mM DTT and 0·08 × SSC, washed in pure ethanol and allowed to dry. These slides were then dipped in photographic emulsion (Kodak NTB-2; IBI Ltd, Cambridge, Cambs, UK) and stored at 4 °C for 18 days in the dark. After being developed (Kodak D-19) and fixed (Kodak Unifix) at 15 °C in the dark, the slides were washed in water, counterstained in haematoxylin, dehydrated through graded alcohols and mounted in Pertex mounting medium (Cellpath, Hemel Hempstead, Herts, UK).

Northern blotting

Total RNA was isolated by the method of Chomczynski & Sacchi (1987) using a commercial kit, and its concentration was determined by measuring absorbance at 260 nm. Total RNA (20 μg) was denatured, electrophoresed in a 1·5% formaldehyde–agarose gel and transferred to a nylon membrane (Amersham International plc) by capillary action in 20 × SSC. The RNA was fixed on to membranes by u.v. cross-linkage (Spectronics Corporation, New York, NY, USA). Membranes were prehybridised for 2 h in 15 ml hybridisation buffer (0·5 M sodium phosphate, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS, 6·7% (v/v) deionised formamide). The full-length human TIMP-1 cDNA probe was labelled with 50 μCi [^{32}P]dCTP by the random priming method, using a commercial kit (Amersham International plc), and added to the hybridisation buffer. Hybridisation was performed overnight at 65 °C.

The membranes were washed twice at 65 °C with 2 × SSC for 15 min and once more with 2 × SSC/0·1% SDS at 65 °C for 15 min (Duncan *et al.* 1996). The blots were laid down to a phosphor screen for 48 h and visualised using a Phosphorimager computer (Molecular Dynamics, Maidstone, Kent, UK). To confirm accurate loading of RNA, the blots were stripped in stripping buffer (5 mM Tris, 0·3 mM EDTA, 0·1 × Denhardt's reagent) for 2 h at 65 °C. The blots were then reprobed with a ^{32}P end-labelled oligonucleotide which hybridises to 18S RNA as described previously (Brooks *et al.* 1992).

Analysis of sections

Sections were viewed by dark-field microscopy and analysed after image capture by computer-aided image analysis (NIH Image 1·55; NIH, Bethesda, MD, USA). To investigate the grain distribution over the corpus luteum, only fixed sections, which had undergone *in situ* hybridisation in carefully controlled conditions in the same run, were analysed. The corpora lutea in each section were identified and the grain density in five fields was analysed by the same observer, blinded to the tissue identity, using a technique of stratified random sampling and monitoring the running mean. Any acellular areas or areas of the section outwith the corpus luteum were ignored. This was repeated at a later date by the same observer to confirm the reproducibility of the results. As TIMP-1 is highly expressed in the corpus luteum, the area proportion of grains was measured, after binary conversion, rather than the absolute grain count. Differences in the area proportion of grains between different groups were investigated by one-way ANOVA. Where an overall statistically significant difference at the 5% level was detected, pairwise comparisons between groups were performed using Bonferroni/Dunn multiple range tests.

As TIMP-1 was also localised to the follicle, the expression of TIMP-1 was analysed in each follicle. All of the different ovarian sections, fixed and frozen, were analysed after *in situ* hybridisation. In order to obtain an idea of the localisation of TIMP-1 as the follicle developed, a technique was devised to classify individual follicles in accordance with their size. Each follicle was measured in light field using an eyepiece graticule calibrated using a standard micrometer slide. The maximal dimension of the follicle from the outside edge of the thecal layer was calculated. As grains were only seen in follicles larger than 200 μm, only these follicles were counted. These were classified as (i) pre-antral, if there was no antrum visible in the plane of section, (ii) antral, if an antrum was visible and the follicle measured less than 600 μm, (iii) small, if the follicle measured less than 1 mm, (iv) medium, if the follicle measured less than 1·5 mm and (v) large, if the follicle measured 1·5 mm or more. The appearance of each follicle was then classified by morphology as normal or atretic (Grimes *et al.* 1987). The grain distribution over the thecal and granulosal layer of each follicle was observed in dark field of the same slides and recorded as absent or present.

Results

TIMP-1 in the corpus luteum

Progesterone concentrations in the control animals were 330 ± 69 nmol/l (mean ± S.E.M.). Functional luteal regression was observed in all animals treated with either the GnRH antagonist or the prostaglandin analogue, as described previously (Fraser *et al.* 1995*b*). Prostaglandin treatment resulted in a decline in progesterone concentrations to 20 ± 5 nmol/l after 12 h and 22 ± 6 nmol/l after 24 h, and treatment with GnRH antagonist resulted

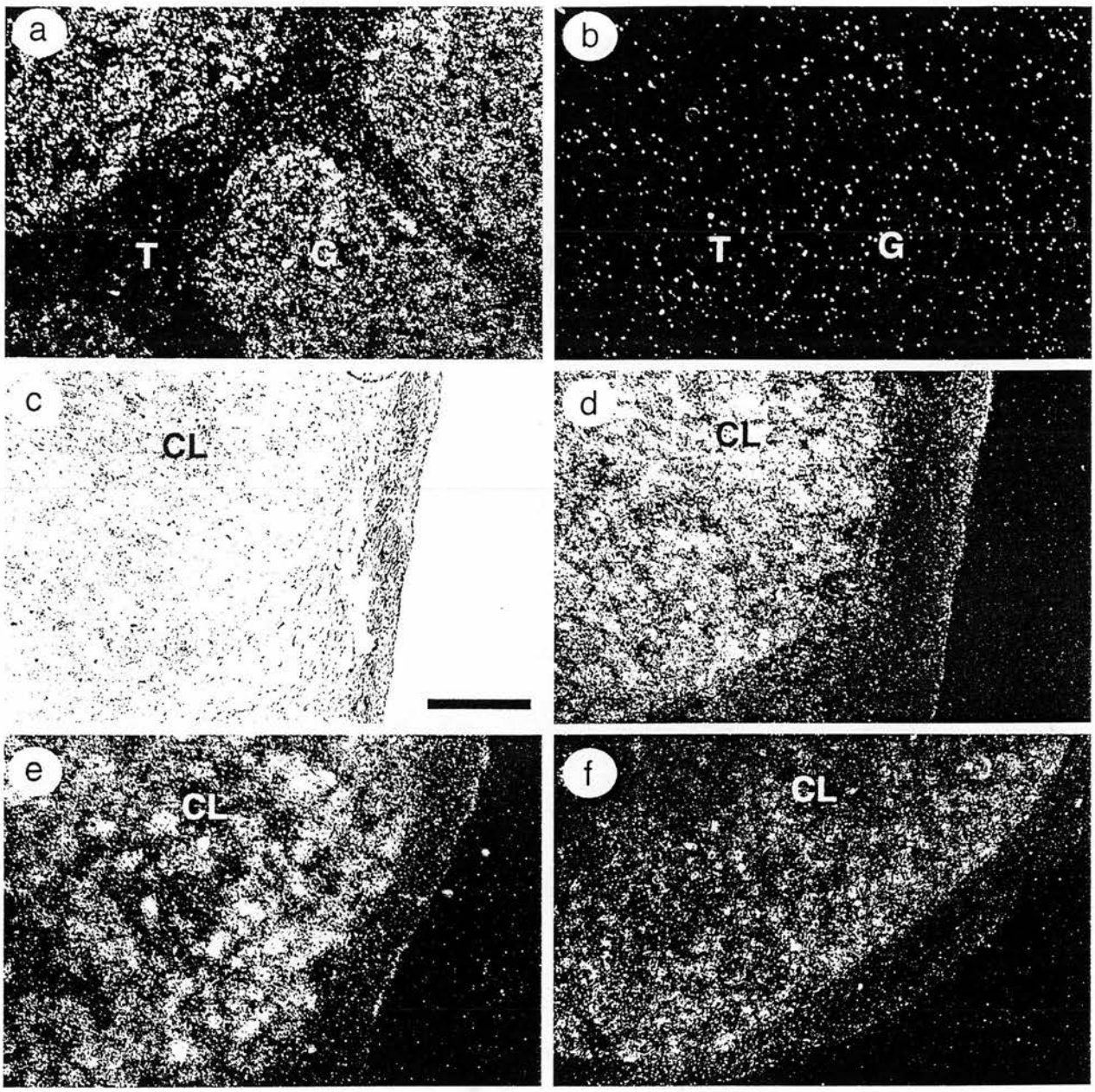

Figure 1 *In situ* hybridisation for TIMP-1 mRNA in the primate corpus luteum. (a) Dark field of TIMP-1 mRNA in the mid-luteal human corpus luteum; many more grains are seen over the granulosa-lutein cells (G) than the theca-lutein cells (T). (b) Dark field negative control serial section of (a) showing very few grains with no difference between the granulosa-lutein cells (G) and the theca-lutein cells (T). (c) Light field of a mid-luteal marmoset ovary showing the position of the corpus luteum (CL). (d) Dark field of section (c) showing TIMP-1 grains localised to the corpus luteum (CL). (e) Dark field of TIMP-1 mRNA in the corpus luteum (CL) of a marmoset ovary 12 h after treatment with a GnRH antagonist. (f) Dark field of TIMP-1 mRNA in the marmoset corpus luteum (CL) 24 h after GnRH antagonist treatment. (Scale bar=200 μm.)

in progesterone concentrations of 13 nmol/l after 12 h and 23 ± 11 nmol/l after 24 h. All progesterone concentrations after induced luteolysis were within the normal range of follicular phase levels in the marmoset (Smith *et al.* 1990).

TIMP-1 mRNA was expressed in the granulosa-lutein cells of the human corpus luteum (Fig. 1a). The human corpus luteum had been included as a positive control as it expresses large amounts of TIMP-1 (Duncan *et al.* 1996).

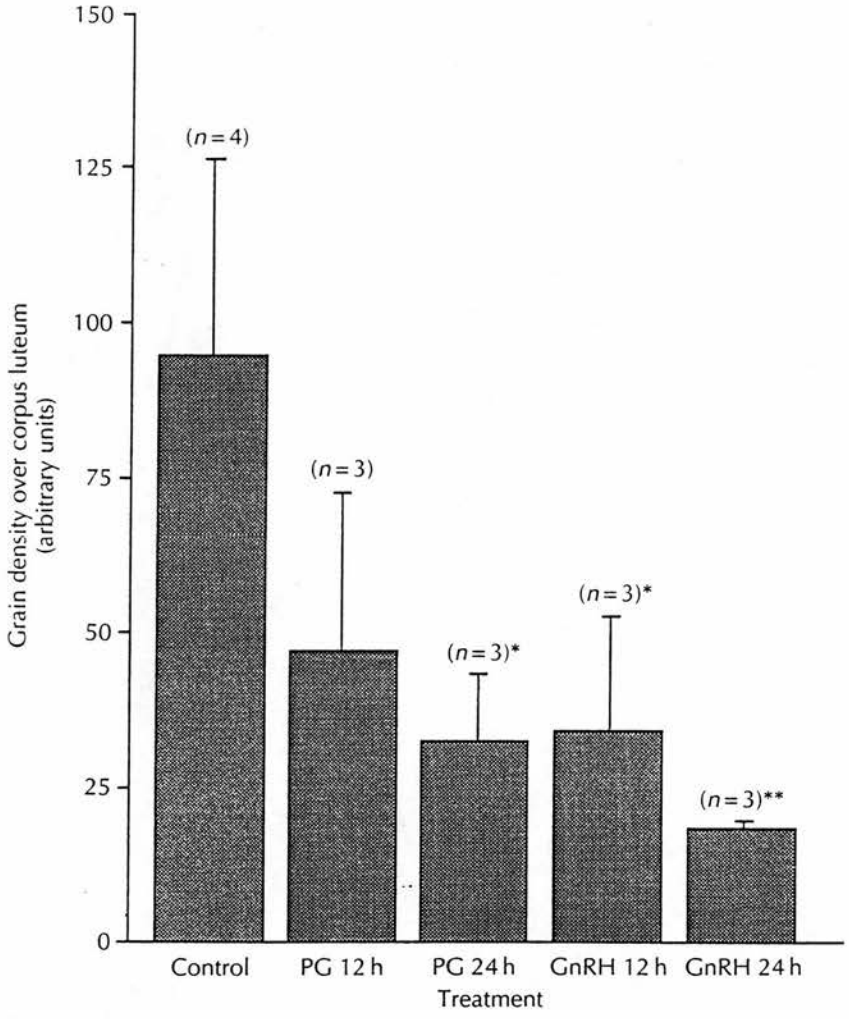

Figure 2 TIMP-1 mRNA in the marmoset corpus luteum after induced luteolysis as measured by grain density after *in situ* hybridisation. Luteolysis had been induced by prostaglandin $F_{2\alpha}$ analogue (PG) or GnRH antagonist (GnRH), 12 or 24 h previously. Values are means ± S.E.M. Significant differences from the control group are indicated for each experimental group: *$P<0.05$; **$P<0.01$.

This grain distribution was absent from all negative control sections, which had been incubated with the sense probe (Fig. 1b).

TIMP-1 message was localised to the corpus luteum in luteal phase marmoset ovaries (Fig. 1c and d). TIMP-1 mRNA could also be localised to the cells of the corpora lutea, 12 and 24 h after induced luteolysis (Fig. 1e and f). However, the silver grains over the corpus luteum were lower in number and more patchily distributed after induced luteolysis (Fig. 1e and f). The area proportion of grains decreased significantly after both prostaglandin ($P<0.05$) and GnRH antagonist ($P<0.05$) treatments (Fig. 2). When specific time points were analysed, grain density was significantly lower 24 h after prostaglandin treatment ($P<0.05$) and both 12 ($P<0.05$) and 24 h ($P<0.01$) after GnRH antagonist treatment (Fig. 2). Treatment with GnRH antagonist or prostaglandin analogue produced similar changes in the appearance of the corpora lutea and the localisation of TIMP-1 mRNA.

TIMP-1 in the follicle

TIMP-1 mRNA was also identified in ovarian follicles (Fig. 3a and b). TIMP-1 expression was absent from the oocyte, the primordial follicle and the small pre-antral follicle less than 200 μm in diameter. Follicles 200 μm or more in diameter expressed TIMP-1 in the thecal cell layer (Fig. 3a, b, c and d). In the healthy follicles, TIMP-1 was absent from the granulosal cell layer at this

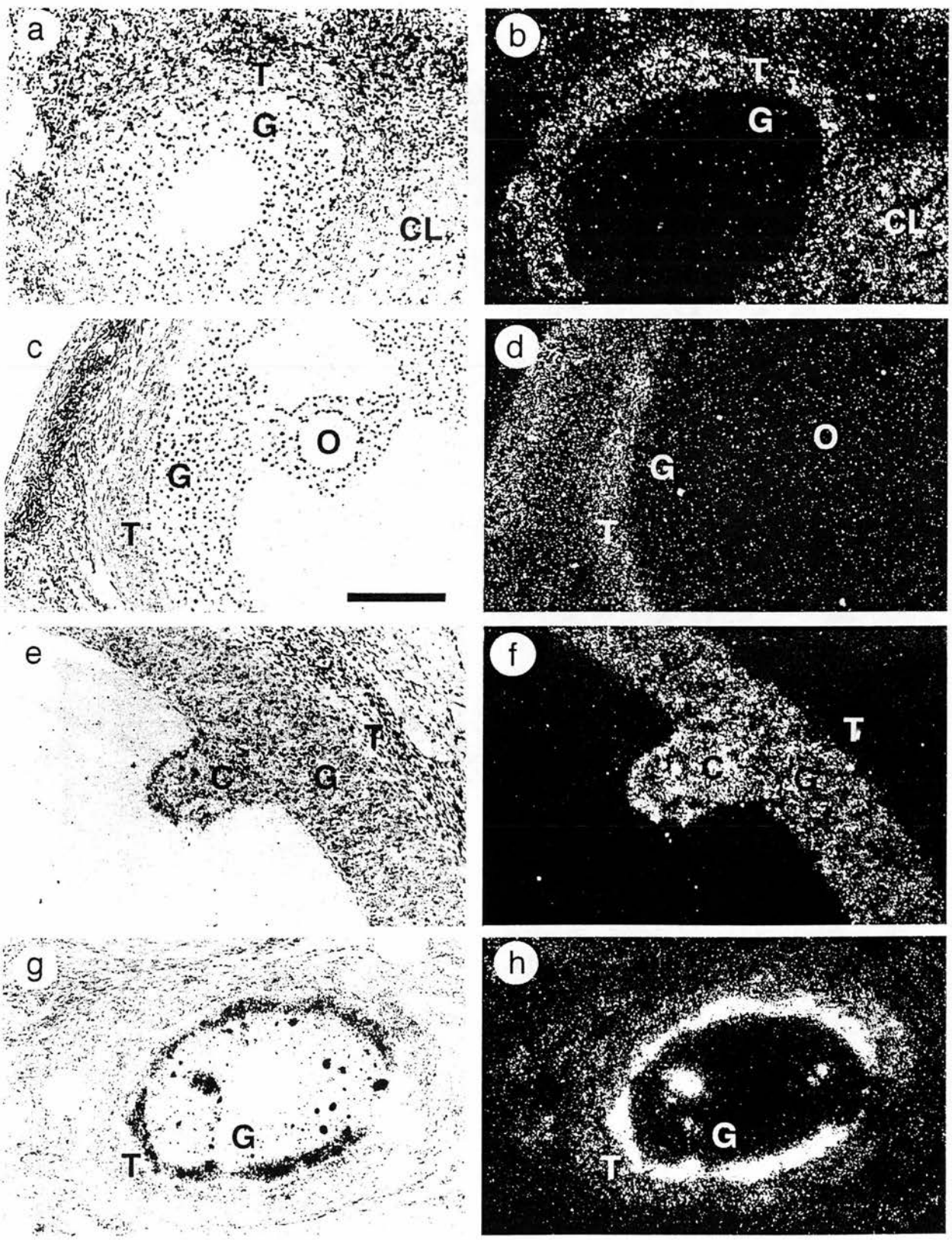
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Table 1 Localisation of TIMP-1 message in marmoset follicles after *in situ* hybridisation. The grain distribution over the thecal and granulosal layers of each follicle larger than 200 μm was recorded. The numbers are the number of follicles with grains over the thecal layer only, the granulosal layer only, no specific grains, grains over both layers and grains over both layers with particular localisation to the granulosal–thecal interface. Follicles were classified as atretic or normal and normal follicles were classified by size as pre-antral/antral (<600 μm), small (<1 mm), medium (<1.5 mm) or large (≥1.5 mm). Asterisks indicate the modal category for each follicle class

	Localisation of TIMP-1 message				
	None	Thecal	Granulosal	Both	Interface
Pre-antral	4	19*	0	0	1
Antral	0	47*	1	2	1
Small	0	43*	2	2	1
Medium	0	33*	1	6	1
Large	0	0	9*	2	0
Atretic	1	0	0	2	31*

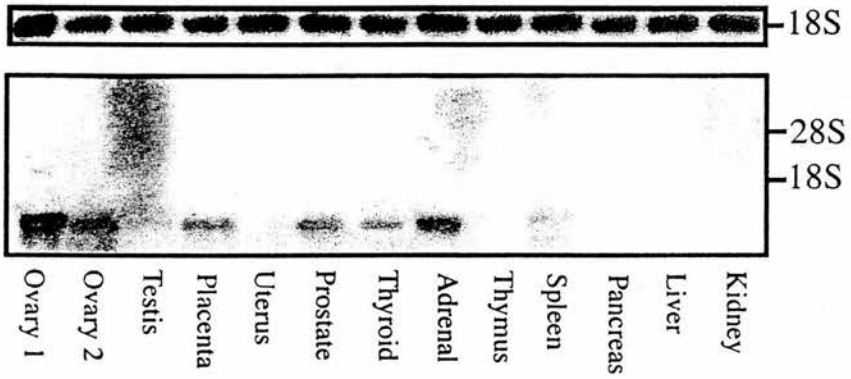

Figure 4 Northern blot of TIMP-1 mRNA in different organs from the marmoset. The positions of the ribosomal 28S and 18S bands are indicated. The 18S bands are shown to demonstrate equal RNA loading between lanes. Ovary 1 was from the luteal phase and ovary 2 was from the follicular phase of the ovarian cycle.

stage. In larger pre-ovulatory follicles (≥1·5 mm) TIMP-1 was noted to be absent from the thecal layer and present in large amounts in the granulosal cell layer (Fig. 3e and f). When the follicle was atretic, a specific localisation of TIMP-1 was noted. TIMP-1 mRNA was found to be localised to thecal cells and individual granulosal cells, and was intensely expressed at the thecal–granulosal interface (Fig. 3g and h). The changing pattern of TIMP-1 localisation noted in the follicle was remarkably consistent in all the follicles analysed (Table 1). The pattern of TIMP-1 expression by the follicles of different sizes appeared to be independent of the stage of the cycle at which the ovary was obtained and was thus identical in ovaries obtained from the follicular phase, the luteal phase or following induced luteolysis.

Figure 3 *In situ* hybridisation for TIMP-1 mRNA in marmoset follicles. (a) Light field of a normal luteal phase marmoset ovary showing a small follicle, with its granulosal (G) and thecal (T) layers next to a corpus luteum (CL). (b) Dark field of section (a) showing localisation of TIMP-1 mRNA by *in situ* hybridisation; grains are seen over the thecal cells (T) and corpus luteum (CL) but are absent from the granulosal cells (G). (c) Light field of a follicular phase ovary showing a medium-sized follicle with the thecal cells (T), granulosal cells (G) and oocyte (O) clearly visible. (d) Dark field of section (c) showing TIMP-1 localised to the thecal cells (T) but absent from the granulosal cells (G) and the oocyte (O). (e) Light field of large pre-ovulatory follicle taken from another follicular phase marmoset ovary showing the thecal layer (T), the granulosal layer (G) and the cumulus granulosa cells (C) surrounding the oocyte. (f) Dark field of section (e) showing TIMP-1 mRNA grains over the granulosa (G) and cumulus cells (C) but absent from the thecal cells (T). (g) Light field of an atretic follicle in an ovary collected 12 h after treatment with prostaglandin $F_{2\alpha}$ analogue, showing the thecal (T) and granulosal (G) layers. (h) Dark field of section (g) showing some TIMP-1 expression in the thecal cells (T), patchy expression in the granulosal cells (G) and marked expression at the thecal–granulosal interface. (Scale bar=200 μm.)

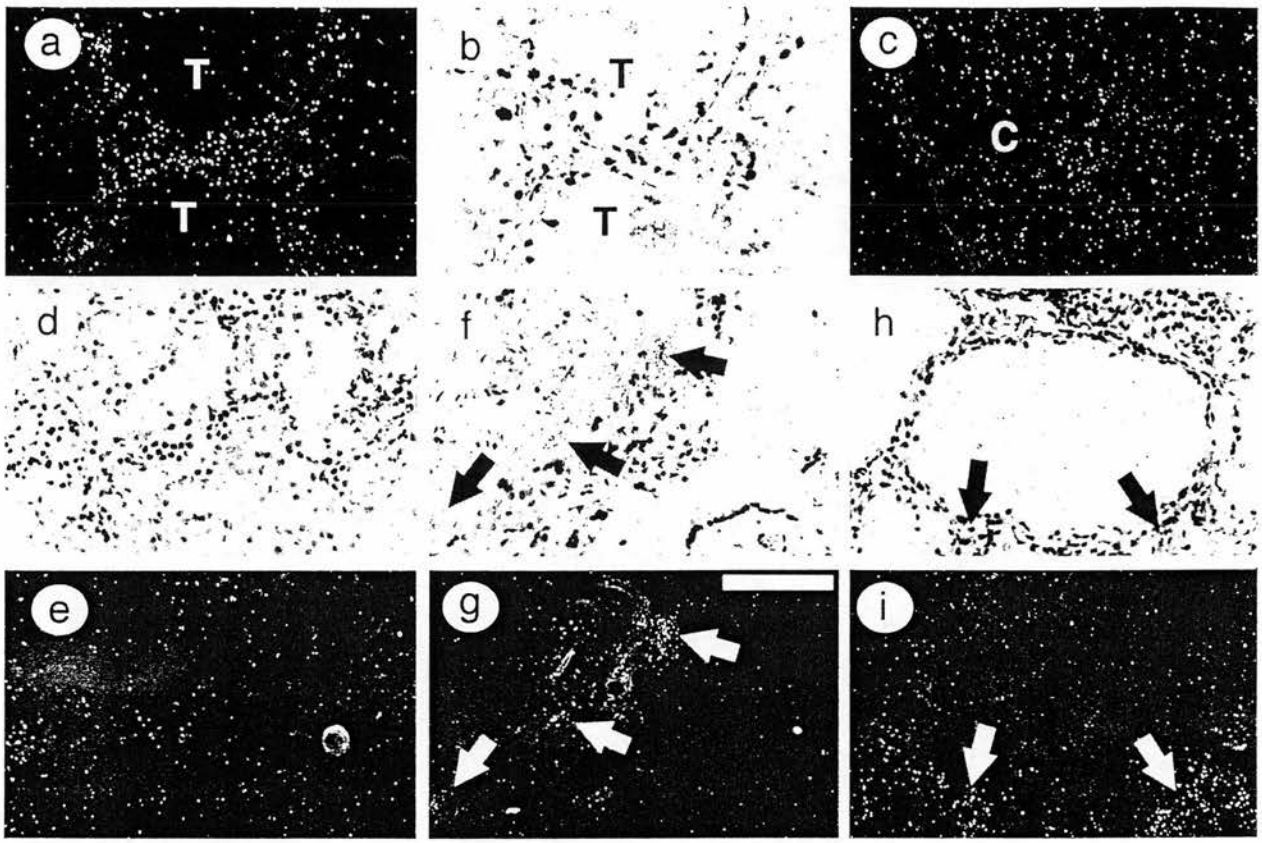

Figure 5 *In situ* hybridisation for TIMP-1 mRNA in various primate organs. (a) Dark field of marmoset testis after *in situ* hybridisation for TIMP-1 with grains seen at the outside of the tubules (T). (b) Light field of (a) showing the tubules (T) and the basal Sertoli cells. (c) Dark field of the marmoset adrenal gland after TIMP-1 *in situ* hybridisation showing a general increased grain distribution over the cortex (C). (d) Light field of the marmoset kidney. (e) Dark field of section (d) showing few grains over the tissue. (f) Light field of the placenta with cells at the periphery of the trophoblast layer highlighted (arrows). (g) Dark field of section (f) showing TIMP-1 to be localised to specific areas of the section (arrows). (h) Light field of the marmoset thyroid gland with some of the parafollicular cells highlighted (arrows). (i) Dark field of section (h) showing TIMP-1 to be localised to these parafollicular cells (arrows). (Scale bar=100 μm.)

TIMP-1 in other tissues

TIMP-1 was found to be expressed in a wide range of marmoset tissues (Fig. 4). A single band, approximately 0·9 kb in length, was detected in total RNA extracted from marmoset tissues. This size is consistent with that reported for human TIMP-1 mRNA (Rapp *et al.* 1990, Duncan *et al.* 1996). The highest levels of expression were seen in steroidogenic tissues, particularly the ovary, adrenal and placenta. Other tissues had low levels of TIMP-1 expression, although specific expression was noted in the thyroid and prostate glands. However, when the localisation of TIMP-1 message was investigated in these tissues by *in situ* hybridisation, the grain density was found to be much less than that seen in the ovary. It became apparent, using *in situ* hybridisation, that TIMP-1 was also expressed in the testis, specifically in the Sertoli cells and interstitial cells (Fig. 5a and b). Specific hybridisation was seen over the steroidogenic cells of the adrenal cortex (Fig. 5c), but not the adrenal medulla. Very few grains were seen over the kidney (Fig. 5d and e), myometrium and endometrium of the uterus, endocrine and exocrine cells of the pancreas, spleen, liver and thymus. Specific areas of TIMP-1 expression were seen in the placenta (Fig. 5f and g), in the parafollicular cells of the thyroid (Fig. 5h and i) and in the glandular cells of the prostate.

Discussion

This is the first study to localise the expression of TIMP-1 mRNA in whole primate ovaries. In common with previously studied species such as the human (Duncan *et al.* 1996) and the ruminant (Smith G W *et al.* 1994), the marmoset corpus luteum expresses a large amount of TIMP-1 message. As TIMP-1 protein synthesis has been demonstrated in these species (Smith *et al.* 1993, Juengel *et al.* 1994, Duncan *et al.* 1996), it is likely that TIMP-1 is

also a major secretory product of the marmoset corpus luteum.

Induction of luteolysis resulted in a marked fall in the level of TIMP-1 expression in the marmoset corpus luteum. The mechanism and control of this fall and its role in tissue remodelling is unclear. The fall in TIMP-1 expression may facilitate increased metalloproteinase digestion of the luteal matrix during regression. However, although rat ovaries and human corpora lutea express metalloproteinases (Endo *et al.* 1993, Duncan *et al.* 1995), and TIMP-2 is also expressed in the ovine corpora lutea (Smith *et al.* 1995), detailed information about the expression of these factors, their localisation, and relationship to TIMP-1 during induced luteolysis is not yet available.

The timing of the fall in TIMP-1 expression in association with the drop in progesterone concentration would also be consistent with a recently proposed steroidogenic role for TIMP-1 (Boujrad *et al.* 1995). There is, however, no indication from these data as to whether the observed changes in TIMP-1 expression are a cause or a consequence of the reduced steroid synthesis. A further consideration is that significant changes in luteal morphology can be seen by 24 h after induced luteal regression in the marmoset (Fraser *et al.* 1995*a*). The fall in TIMP-1 expression may therefore be related to the death of viable luteal cells in the corpus luteum at this time (Fraser *et al.* 1995*c*).

These findings are contrary to previous observations of the effects of induced luteolysis in the cow (Juengel *et al.* 1994). TIMP-1 expression in the bovine corpus luteum was found to increase up to 24 h after prostaglandin $F_{2\alpha}$ injection and then return to pretreatment levels within 48 h. This occurs at a time when increasing cell death is observed in the bovine corpus luteum (Juengel *et al.* 1993). We have examined TIMP-1 expression at 12 and 24 h after induced luteolysis and found a marked and persistent fall in TIMP-1 expression. However, the luteolytic processes of the primate and the ruminant are different in both nature and time span (Auletta & Flint 1988) and this study provides further evidence of these differences.

We have shown that TIMP-1 is also expressed by follicles in the primate ovary and that the localisation changes relative to the apparent size of the follicle. These data are limited in that two-dimensional interpretation of three-dimensional pre-ovulatory follicles may lead to an underestimation of the size and nature of some follicles. Volume changes may also result from processing and sectioning of the tissue. In spite of these deficiencies, a consistent pattern was observed in the follicular localisation of TIMP-1 mRNA relative to the size of the follicle.

TIMP-1 is first expressed in the thecal cell layer of pre-antral follicles which are larger than 200 μm in diameter. This is the stage when the follicle becomes gonadotrophin-dependent (Zeleznik & Fairchild Benyo 1994) and LH receptors can first be detected in the theca (Richards & Midgely 1976). In the rat, LH stimulates the secretion of TIMP-like protein and increases TIMP-1 mRNA (Mann *et al.* 1991, Morgan *et al.* 1994). It is therefore possible that TIMP-1 expression in the thecal cells is related to the action of LH. In the testis, TIMP-1 production by the Sertoli cells is induced by FSH (Ulisse *et al.* 1994, Boujrad *et al.* 1995). Although the granulosal cells express FSH receptors at this stage and respond to FSH (Zeleznik & Fairchild Benyo 1994), they do not express TIMP-1 mRNA. It is therefore likely that the control of TIMP-1 expression by FSH is different in the ovarian follicle and the testicular tubule.

In contrast, all the large pre-ovulatory follicles ($\geq 1{\cdot}5$ mm) expressed TIMP-1 in the granulosal cells. Smith *et al.* (1994*a*) showed that the ovulatory gonadotrophin surge induced granulosal cell TIMP-1 expression in the sheep. Our results are consistent with a similar pre-ovulatory induction of TIMP-1 in the granulosa cells of the primate ovary. As the granulosa cells express the LH receptor at this stage (Richards & Midgely 1976), the increased TIMP-1 expression may be directly induced by LH. It is not clear why the thecal cells stop expressing TIMP-1, but this pattern of TIMP-1 expression is seen in the granulosa-derived and the theca-derived cells of the human corpus luteum throughout its functional lifespan (Duncan *et al.* 1996).

The expression of TIMP-1 in the atretic follicles was very different. This was independent of luteolytic treatment or stage of the ovarian cycle. There was a patchy expression in the thecal and granulosal cell layers but marked expression around the basal lamina at the cellular interface. During follicular atresia, the steroidogenic cells degenerate (Hay *et al.* 1976) and the basal lamina breaks down (Bagavandoss *et al.* 1983). These data are consistent with a role for matrix metalloproteinase involvement in the tissue remodelling associated with follicular atresia. The expression of TIMP-1 may be induced to control local metalloproteinases involved in the breakdown of the basal lamina. However, the expression of metalloproteinases in the atretic follicle has not yet been described, but they have been shown to be expressed during the breakdown of the follicle wall during ovulation (Reich *et al.* 1985, Russell *et al.* 1995).

The physiological role of TIMP-1 in the steroidogenic cells of the follicle is not clear. It is likely that it is involved in the regulation of the matrix remodelling which occurs during follicular growth and development. TIMP-1 has been shown to have proliferative effects on cells *in vitro* (Hayakawa *et al.* 1992) but it remains to be established whether it can function as a growth factor *in vivo*. In addition, the pattern of TIMP-1 expression is similar to the localisation of several steroidogenic enzymes in different sizes of follicle (Richards *et al.* 1995). This is consistent with a role for TIMP-1 as a steroidogenic agent.

TIMP-1 was also found to be expressed in other tissues. This is not surprising as TIMP-1 is involved in tissue remodelling throughout the body (Salamonsen 1996). The luteal phase ovary expressed far higher levels of TIMP-1 mRNA than any of the other tissues investigated. Expression in the placenta and adrenal has previously been noted in the ewe (Hampton *et al.* 1995). TIMP-1 expression has previously been reported in human endometrium (Hampton & Salamonsen 1994) but we did not detect it in the marmoset endometrium. This may be because the marmoset monkey does not have a menstrual cycle and lacks cyclical endometrial remodelling. Sertoli cell expression of TIMP-1 has been reported in the rat (Boujrad *et al.* 1995). As the vast majority of total testicular RNA is of germ cell origin, we were best able to confirm its expression in the marmoset testis by *in situ* hybridisation.

TIMP-1 was particularly expressed by steroidogenic tissues such as the ovary, placenta, testis and adrenal. This expression was generally localised to the steroidogenic cells of these organs. Although the ovary and placenta undergo extensive remodelling during their lifespan, the adult adrenal and testis do not. Whether TIMP-1 has any role in these tissues other than specific inhibition of metalloproteinases has yet to be determined. However, these data are consistent with an additional role for TIMP-1 as a facilitator of steroidogenesis *in vivo*. The ontogeny and the role of TIMP-1 in endocrine glands requires further investigation and functional studies of TIMP-1 are required.

In conclusion, the fall in TIMP-1 expression in association with primate luteolysis and its changing localisation during the lifespan of the follicle support a role for modulation of TIMP-1 in the control of tissue remodelling. In addition, these data and the predominant expression of TIMP-1 in steroidogenic tissues support the concept that TIMP-1 has an additional role as a facilitator in steroidogenesis.

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References

Auletta FJ & Flint APF 1988 Mechanisms controlling corpus luteum function in sheep, cows, nonhuman primates, and women especially in relation to the time of luteolysis. *Endocrine Reviews* **9** 88–105.

Bagavandoss P, Midgley AR Jr & Wich M 1983 Developmental changes in the ovarian follicular basal lamina detected by immunofluorescence and electron microscopy. *Journal of Histochemistry and Cytochemistry* **31** 633–640.

Behrman HR, Endo T, Aten RF & Musicki B 1993 Corpus luteum function and regression. *Reproductive Medicine Review* **2** 153–180.

Birkedal-Hansen H 1995 Proteolytic remodeling of the extracellular matrix. *Current Opinion in Cell Biology* **7** 728–735.

Boujrad N, Ogwuegbu SO, Garnier M, Lee C-H, Martin BM & Papadopoulos V 1995 Identification of a stimulator of steroid hormone synthesis isolated from testis. *Science* **268** 1609–1612.

Brooks J, Crow WJ, McNeilly JR & McNeilly AS 1992 Relationship between gonadotropin subunit gene-expression, gonadotropin-releasing hormone receptor content and pituitary and plasma gonadotropin concentrations during the rebound release of FSH after treatment of ewes with bovine follicular-fluid during the luteal phase of the cycle. *Journal of Molecular Endocrinology* **8** 109–118.

Chomczynski P & Sacchi N 1987 Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.

Deghenghi R, Boutignon F, Wuthrich P & Lenaerts V 1993 Antarelix (EP 24332) a novel water soluble LHRH antagonist. *Biomedicine and Pharmacology* **47** 107–110.

Duncan WC, McNeilly AS & Illingworth PJ 1995 The effect of luteal rescue on the expression of matrix metalloproteinases and their inhibitors in the human corpus luteum. *Biology of Reproduction* **52** Suppl 1 222.

Duncan WC, McNeilly AS & Illingworth PJ 1996 Expression of tissue inhibitor of metalloproteinases-1 in the human corpus luteum after luteal rescue. *Journal of Endocrinology* **148** 59–67.

Endo T, Aten RF, Wang F & Behrman HR 1993 Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. *Endocrinology* **133** 690–698.

Fraser HM, Lunn SF, Cowen GM & Illingworth PJ 1995*a* Induced luteal regression in the primate: evidence for apoptosis and changes in *c-myc* protein. *Journal of Endocrinology* **147** 131–137.

Fraser HM, Lunn SF, Whitelaw PF & Hillier SG 1995*b* Induced luteal regression: differential effects on follicular and luteal inhibin/activin subunit mRNAs in the marmoset monkey. *Journal of Endocrinology* **144** 201–208.

Fraser HM, Lunn SF, Young FM, Eidne KA, Sellar R, Illingworth PJ & Harrison DJ 1995*c* Apoptosis and necrosis after induced luteal regression in the primate. *Biology of Reproduction* **52** Suppl 1 383.

Grimes RW, Malton P & Ireland JJ 1987 A comparison of histological and non-histological indices of atresia and follicular function. *Biology of Reproduction* **37** 82–88.

Hampton AL & Salamonsen LA 1994 Expression of messenger ribonucleic acid encoding matrix metalloproteinases and their tissue inhibitors is related to menstruation. *Journal of Endocrinology* **141** R1–R3.

Hampton AL, Butt AR, Riley SC & Salamonsen LA 1995 Tissue inhibitors of metalloproteinases in endometrium of ovariectomized steroid-treated ewes and during the estrous cycle and early pregnancy. *Biology of Reproduction* **53** 302–311.

Hay MF, Cran DG & Moor RM 1976 Structural changes occurring during atresia in sheep ovarian follicles. *Cell and Tissue Research* **169** 515–529.

Hayakawa T, Yamashita K, Tanzawa K, Uchijima E & Iwata K 1992 Growth promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. *FEBS Letters* **298** 29–32.

Juengel JL, Garverick HA, Johnson AL, Youngquist RS & Smith MF 1993 Apoptosis during luteal regression in cattle. *Endocrinology* **132** 249–254.

Juengel JL, Smith GW, Smith MF, Youngquist RS & Garverick HA 1994 Pattern of protein production by bovine corpora lutea during

luteolysis and characterization of expression of two major secretory products of the regressing corpora lutea. *Journal of Reproduction and Fertility* **100** 515–520.

Luck MR & Zhao Y 1995 Structural remodelling of reproductive tissues. *Journal of Endocrinology* **146** 191–195.

Mann JS, Kindy MS, Edwards DR & Curry TE Jr 1991 Hormonal regulation of matrix metalloproteinase inhibitors in rat granulosa cells and ovaries. *Endocrinology* **128** 1825–1832.

Matrisian LM 1990 Metalloproteinases and their inhibitors in matrix remodelling. *Trends in Genetics* **6** 121–125.

Morgan A, Keeble SC, London SN, Mase KN & Curry TE Jr 1994 Antiprogesterone (RU 486) effects on metalloproteinase inhibitor activity in human and rat granulosa cells. *Fertility and Sterility* **61** 949–955.

Rapp G, Freudenstein J, Klaudiny J, Mucha J, Wempe F, Zimmer M & Scheit KH 1990 Characterisation of three abundant mRNAs from human ovarian granulosa cells. *DNA and Cell Biology* **9** 479–485.

Reich R, Tsafriri A & Mechanic GL 1985 Involvement of collagenolysis in ovulation in the rat. *Endocrinology* **116** 522–527.

Richards JS & Midgley AR Jr 1976 Protein hormone action: a key to understanding ovarian follicular and luteal cell development. *Biology of Reproduction* **14** 82–94.

Richards JS, Fitzpatrick SL, Clemens JW, Morris JK, Alliston T & Sirois J 1995 Ovarian cell differentiation: a cascade of multiple hormones, cellular signals and regulated genes. *Recent Progress in Hormone Research* **50** 223–254.

Russell DL, Salamonsen LA & Findlay JK 1995 Immunization against the N-terminal peptide of the inhibin α43-subunit (αN) disrupts tissue remodeling and the increase in matrix metalloproteinase-2 during ovulation. *Endocrinology* **136** 3657–3664.

Salamonsen LA 1996 Matrix metalloproteinases and their tissue inhibitors in endocrinology. *Trends in Endocrinology and Metabolism* **7** 28–34.

Smith KB, Lunn SF & Fraser HM 1990 Inhibin secretion during the ovulatory cycle and pregnancy in the common marmoset monkey. *Journal of Endocrinology* **126** 489–495.

Smith GW, Moor RM & Smith MF 1993 Identification of a 30 000 M_r polypeptide secreted by cultured ovine granulosa cells and luteal tissue as a tissue inhibitor of metalloproteinases. *Biology of Reproduction* **48** 125–132.

Smith GW, Goetz TL, Anthony RV & Smith MF 1994*a* Molecular cloning of an ovine ovarian tissue inhibitor of metalloproteinases: ontogeny of messenger ribonucleic acid expression and *in situ* localization within preovulatory follicles and luteal tissue. *Endocrinology* **134** 344–352.

Smith MF, Kemper CN, Smith GW, Goetz TL & Jarrell VL 1994*b* Production of tissue inhibitor of metalloproteinases-1 by porcine follicular and luteal cells. *Journal of Animal Science* **72** 1004–1012

Smith GW, McCrone S, Petersen SL & Smith MF 1995 Expression of messenger ribonucleic acid encoding tissue inhibitor of metalloproteinases-2 within ovine follicles and corpora lutea. *Endocrinology* **136** 570–576.

Ulisse S, Farina AR, Piersanti D, Tiberio A, Cappabianca I, Dorazi G, Jannini EA, Malykh O, Stetler-Stevenson WG, Darmiento M, Gulino A & Mackay AR 1994 Follicle-stimulating hormone increases the expression of tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 and induces TIMP-1 AP-1 site binding complex(es) in prepubertal rat Sertoli cells. *Endocrinology* **135** 2479–2487.

Zeleznik AJ & Fairchild Benyo DF 1994 Control of follicular development, corpus luteum function, and the recognition of pregnancy in higher primates. In *The Physiology of Reproduction*, edn 2, pp 751–780. Eds E Knobil & JD Neill. New York: Raven Press.

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The Effect of Luteal "Rescue" on the Expression and Localization of Matrix Metalloproteinases and Their Tissue Inhibitors in the Human Corpus Luteum

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ABSTRACT

Luteolysis is associated with tissue remodeling probably involving the matrix metalloproteinases (MMPs) and their specific tissue inhibitors (TIMPs). This study investigated the expression and localization of the major MMPs and TIMPs in the human corpus luteum throughout the luteal phase and after luteal rescue with hCG. Corpora lutea (n = 9) were collected at hysterectomy and were dated by serial urinary LH estimation. In addition, corpora lutea (n = 3) were collected from women who had received daily doubling doses of hCG to mimic the hormonal changes of early pregnancy. MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP-3 were investigated by zymography, reverse zymography, Northern blotting, and *in situ* hybridization. There was no change in the expression of MMP-1, TIMP-1, and TIMP-2 throughout the luteal phase or after luteal rescue. Little TIMP-3 could be detected in the corpus luteum. MMP-9 activity peaked in the early and late luteal phase. The expression and activity of MMP-2 were maximal in the late luteal phase. Exposure to hCG during luteal rescue *in vivo* was associated with a reduction ($P < 0.05$) in the expression and activity of MMP-2. Messenger ribonucleic acids (mRNAs) for MMP-1, MMP-2, and TIMP-2 were localized to the connective tissue stroma and the thecal-lutein cells of the corpus luteum. In contrast, TIMP-1 mRNA was localized to the granulosa-lutein cells, and MMP-9 mRNA was expressed in scattered cells within the steroidogenic and nonsteroidogenic cell layers. In conclusion, during maternal recognition of pregnancy, hCG prevents the normal increase in MMP-2 in the late luteal phase. MMPs can function in an environment containing large amounts of TIMP-1, as they have a different cellular localization. (*J Clin Endocrinol Metab* **83:** 2470–2478, 1998)

UNLESS hCG is secreted from the implanting blastocyst, the human corpus luteum will undergo structural and functional luteolysis (1). The corpus luteum changes from the most active endocrine gland in the body, with a blood flow per unit mass much greater than that of the kidney (2), to a small fibrous remnant in a matter of days. This extensive tissue remodeling is likely to involve a group of zinc-dependent proteolytic enzymes known as the matrix metalloproteinases (MMPs) (3–5). These enzymes have been implicated in a wide variety of biological processes that involve remodeling of the extracellular matrix (ECM), such as ovulation, menstruation, angiogenesis, and tumor growth and metastasis (6–8).

The activity of MMPs is controlled at several levels, including synthesis as proenzymes, enzyme activation, and the production of specific tissue inhibitors (9, 10). Tissue inhibitors of metalloproteinases (TIMPs) are of particular interest, as TIMP-1 is one of the major products of the corpus luteum. It is produced in large amounts by the corpus luteum of many species, including the rat (11), sheep (12), cow (13), pig (14), monkey (15), and human (16). In addition, it has recently been reported that TIMP-2 is produced by corpora lutea of rats (11), sheep (17), and cows (18), and that TIMP-3 can also be detected in rat ovaries (11).

TIMPs bind to and inhibit metalloproteinase enzymes with a one to one stoichiometry (10). As TIMP-1, in particular, is produced in large amounts throughout the normal luteal phase (16), it is not clear how metalloproteinase enzymes function in an environment containing large amounts of specific inhibitor. This study aimed to investigate the expression and localization of the common MMPs and their specific tissue inhibitors in the human corpus luteum throughout the normal luteal phase and the effect of luteal rescue with exogenous hCG, to mimic the hormonal changes of early pregnancy.

Materials and Methods

Materials

All reagents were obtained from Sigma Chemical Co. (Poole, UK), unless otherwise stated. Prof. M. R. Waterman of Vanderbilt University (Nashville, TN) provided the antibody to 17α-hydroxylase. The probes to MMP-2 (gelatinase-A), MMP-9 (gelatinase-B), TIMP-1, and TIMP-2 were provided by British Biotech Pharmaceuticals (Oxford, UK). Probes for TIMP-3 and MMP-1 (interstitial collagenase) were purchased from University Technologies International (Calgary, Canada). The reverse zymography kit was also obtained commercially from University Technologies International. All restriction enzymes and ribonucleic acid (RNA) polymerases were obtained from Promega (Southampton, UK). Human placental tissue was obtained from the local maternity hospital.

Collection of corpora lutea

Corpora lutea were enucleated at the time of hysterectomy in women undergoing surgery for benign conditions as described previously (16). In all women, only one corpus luteum was identified on the surface of one of the ovaries. In each case this corpus luteum was removed and

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studied as described below. All women were healthy, aged 32–45 yr, with regular menstrual cycles and had not received any form of hormonal treatment for at least 3 months before taking part in the study. The date of the LH surge was determined by estimation of LH concentrations in serial early morning urine samples collected before operation (19). On this basis, three corpora lutea classified as early luteal (LH+1 to LH+5), three as midluteal (LH+6 to LH+10), and three as late luteal (LH+11 to LH+14) were investigated. In addition, three women were given im injections of hCG (Profasi, Serono Laboratories, Welwyn Garden City, UK) from LH+7 in daily doubling doses, starting at 125 IU, for 6–8 days until surgery. This regimen has been shown to reproduce the hormonal changes of early pregnancy (20). An additional corpus luteum was obtained from a woman who had received hCG for 8 days to achieve luteal rescue, but the operation was postponed. This corpus luteum was collected 3 days after the final hCG injection.

At operation, the whole corpus luteum was enucleated from the ovary by blunt dissection, and the ovary was oversewn. The tissue was immediately divided into radial blocks to ensure that the whole thickness of the gland was represented in any piece. Two pieces of tissue were rapidly snap-frozen in liquid nitrogen and stored at −70 C for subsequent protein and RNA extraction. One piece was frozen in embedding medium (Tissue-Tek OCT compound, Miles, Elkhart, IN) and stored at −70 C. Serial frozen sections (6 μm) were cut onto ribonuclease-free slides coated with poly-L-lysine (50 μg/l) and stored at −70 C until use. In each case, an endometrial biopsy was fixed in 4% paraformaldehyde and processed into paraffin wax for luteal phase dating by tissue morphometry (21). Plasma was taken before surgery, and the progesterone concentration was measured using a standard RIA (22). This study was approved by the Reproductive Medicine Branch of the South-East of Scotland ethics committee, and informed consent was obtained from all patients before tissue collection.

Gelatin zymography

Protein was extracted from corpora lutea in 0.1% (wt/vol) SDS at 4 C. The protein content of the sample after sonication was measured using the method of Bradford (23). Seventy-five micrograms of protein in sample buffer [10% (vol/vol) glycerol, 1% (wt/vol) SDS, and 0.04% (vol/vol) bromophenol blue] were applied, without heating or reduction, to an 11% (wt/vol) polyacrylamide gel containing 1 mg/mL gelatin and 0.1% (wt/vol) SDS. After electrophoretic separation of proteins, the gels were incubated in 2.5% Triton X-100 for 30 min to remove the SDS. The gels were then incubated for 16 h at 37 C in 50 mmol/L Tris-HCl (pH 7.6) containing 0.2 mol/L NaCl, 5 mmol/L $CaCl_2$, and 0.02% (wt/vol) Brij 35. The gels were stained in staining solution [30% (vol/vol) methanol, 10% glacial acetic acid, and 0.5% (wt/vol) Coomassie brilliant blue G250] and then destained in the same solution in the absence of dye.

Reverse zymography

Reverse zymography using 75 μg of each protein sample was performed using a commercial kit. Briefly, 12% (wt/vol) polyacrylamide gels containing 0.1% (wt/vol) SDS, 1 mg/mL gelatin, and a solution of secreted metalloproteinases (as supplied) were prepared. After electrophoresis, the gels were washed overnight in a solution of 2.5% Triton X-100, 50 mmol/L Tris-HCl (pH 7.5), and 5 mmol/L $CaCl_2$. The gels were rinsed in water and incubated in 50 mmol/L Tris-HCl (pH 7.5) and 5 mmol/L $CaCl_2$ with gentle shaking for 24 h at 37 C. Staining and destaining were carried out as described above, and bands corresponding to TIMP-1, TIMP-2, and TIMP-3 were identified by reference to the standards supplied with the kit.

Northern blot analysis

Total cellular RNA was isolated by the method of Chomczynski and Sacchi (24) using a commercial kit, and its concentration was determined by absorption at 260 nm. Total RNA (20 μg) was denatured, electrophoresed in a 1.5% formaldehyde-agarose gel, and transferred to a nylon membrane (Amersham International, Aylesbury, UK) by capillary action in 20 × SSC (1 × SSC is 150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7). Northern blot analysis was conducted as described previously (16) using [^{32}P]deoxy-CTP-labeled complementary DNA probes. The complementary DNA probes were derived from the following plasmids: a 0.7-kb fragment of human MMP-1 in pBluescript, a 1.6-kb fragment (6–1576 bp) of human MMP-2 in pGEM 4Z, a 1.3-kb fragment (759–2105 bp) of human MMP-9 in pGEM4Z, full-length human TIMP-1 in pGEM4Z, full-length human TIMP-2 in pGEM4Z, and a 0.2-kb fragment (400–600 bp) of human TIMP-3 in pBluescript. After washing (16), the blots were laid onto a phosphor screen for 48–72 h and visualized using a PhosphorImager computer (Molecular Dynamics, Maidstone, UK). The blots were then stripped (14) and reprobed with a ^{32}P end-labeled oligonucleotide that hybridizes to 18S RNA, as described previously (25). The molecular size of the bands was calculated with reference to a standard RNA mol wt marker (Promega) run in an adjacent lane.

In situ hybridization

Isotopic *in situ* hybridization was performed on frozen sections using ^{35}S-labeled riboprobes. Antisense and sense riboprobes incorporating ^{35}S-labeled UTP (Amersham International) were synthesized using a commercial kit (Promega). The riboprobes were generated from the above plasmids using the following restriction enzymes and RNA polymerases: MMP-1, *Hin*dIII with T7 polymerase (antisense), and *Not*I with T3 polymerase (sense); MMP-2, *Eco*RI with T7 polymerase (antisense), and *Hin*dIII with SP6 polymerase (sense); MMP-9, *Eco*RI with T7 polymerase (antisense), and *Pst*I with SP6 polymerase (sense); TIMP-1, *Kpn*I with T7 polymerase (antisense), and *Hin*dIII with SP6 polymerase (sense); and TIMP-2, *Hin*dIII with SP6 polymerase (antisense), and *Eco*RI with T7 polymerase (sense).

In situ hybridization was conducted according to the method described previously (15) at 55 C using 1 × 10^6 cpm ^{35}S-labeled antisense riboprobe. The ^{35}S-labeled sense riboprobe (1 × 10^6 cpm) was added to serial sections as a negative control. After washing under increasingly stringent conditions (15), the slides were dipped in photographic emulsion (Kodak NTB-2, IBI, Cambridge, UK) and incubated at 4 C for 21 days in the dark. After developing (Kodak D19) and fixing (Kodak Unifix) at 15 C in the dark, the sections were washed in water, counterstained with hematoxylin, dehydrated through graded alcohols, and mounted (Pertex, Cellpath, Hemel Hempstead, UK).

Immunohistochemistry

Frozen sections were fixed at 4 C in 15% (vol/vol) aqueous picric acid containing 2% (wt/vol) paraformaldehyde, pH 7.4, for 10 min and washed in phosphate-buffered saline for 20 min at 4 C. Nonspecific binding was blocked using a goat serum solution [normal goat serum (SAPU, Carluke, UK) diluted 1:5 in Tris-buffered saline with 5% (wt/vol) BSA]. The primary antibody to 17α-hydroxylase was diluted to a concentration of 1:1500 in Tris-buffered saline and applied to the section for 20 h at 4 C. Antibody binding was visualized with an avidin-biotin-alkaline phosphatase complex (AB-AP kit, Dako, High Wycombe, UK) using biotinylated goat antirabbit Igs (Dako) as the secondary antibody. Coloration was achieved using a substrate that produced a red end product (Alkaline Phosphatase Substrate Kit I, Vector Laboratories, Peterborough, UK). Sections were counterstained with hematoxylin, dehydrated, and mounted as described above.

Analysis of results

The intensities of the 92- and 66-kDa bands detected by zymography were measured by computer-aided densitometric image analysis (NIH Image 1.55, NIH, Bethesda, MD) after image capture and inversion. Northern blot band intensity was measured using the PhosphorImager computer. To correct for minor differences in loading, the ratio of the relative band intensity to the 18S band intensity was used for data analysis. One-way ANOVA was used to investigate differences in expression throughout the luteal phase. The rescued corpora lutea were compared to the late luteal corpora lutea using an unpaired *t* test. A commercial software package was used for statistical analysis (StatView 4.0, Abacus Concepts, Berkeley, CA).

Results

Plasma progesterone concentrations

The classification of the corpora lutea by serial urinary LH measurement agreed with the luteal phase dating of endo-

metrial biopsies using the method of Li *et al.* (21). The plasma progesterone concentrations were 35.3 ± 9.8 nmol/L in the early luteal samples, 41.0 ± 9.9 nmol/L in the midluteal samples, and 19.2 ± 12.9 nmol/L in the late luteal samples. After luteal rescue by exogenous hCG, plasma progesterone concentrations had increased to 52.6 ± 1.5 nmol/L. The plasma progesterone concentration in the postrescue sample was 9.16 nmol/L.

Identification of metalloproteinases and their tissue inhibitors

Three distinct bands of gelatinase activity at 92, 72, and 66 kDa were detected in the human corpus luteum by gelatin zymography (Fig. 1). These are consistent with MMP-9 and the latent and active forms of MMP-2, respectively (4, 26). Reverse zymography demonstrated a band of inhibition of gelatinase activity at approximately 28 kDa and a lighter band at 21 kDa (Fig. 2). These correspond to TIMP-1 and TIMP-2, respectively (4, 27). An additional band at 24 kDa was seen in human placental tissue, but was absent from corpora lutea. This is consistent with TIMP-3 (27), which is produced by decidual tissue (28). TIMP-1 and TIMP-2 could be detected in samples taken from different stages of the luteal phase and after luteal rescue with exogenous hCG (Fig. 2). The activities of MMP-2 and MMP-9 changed over the luteal phase (Fig. 3). MMP-9 activity peaked in the early and late luteal phase and was lowest in the midluteal phase ($P < 0.05$). In contrast, MMP-2 activity increased throughout the luteal phase to a maximum in the late luteal phase ($P < 0.05$). Luteal rescue with hCG resulted in lower MMP-2 activity than during the late luteal phase in the absence of hCG ($P < 0.05$). When the corpus luteum was rescued with hCG, and trophic support was withdrawn (in the postrescue sample), large amounts of MMP-2 activity were clearly identified by zymography (Fig. 1).

Expression of metalloproteinases and their tissue inhibitors

A single band of approximately 0.9 kb corresponding to TIMP-1 (16, 29) was detected in human corpora lutea by Northern blotting (Fig. 4). This confirms our previously reported results (16). Northern blotting for TIMP-2 resulted in a single band of 3.6 kb (Fig. 4). This is consistent with the transcript size for TIMP-2 messenger RNA (mRNA) in the human (30). Several mRNA species corresponding to TIMP-3 (28) were detected in the placenta, but were not seen in the human corpus luteum (data not shown). As we have previously reported (16), there were no significant differences in the level of TIMP-1 expression throughout the luteal phase or after luteal rescue with hCG (Fig. 5). Likewise, TIMP-2 expression did not change throughout the luteal phase or after luteal rescue (Fig. 5).

Specific mRNA transcripts of 3.5 kb were detected in corpora lutea after Northern blotting for MMP-2. This is consistent with the reported transcript size of MMP-2 (31). In addition, Northern blotting for MMP-1 demonstrated transcripts of 3.6 kb and approximately 1.0 kb in human corpora lutea (data not shown). Little MMP-9 expression could be detected by Northern blotting despite clear identification by zymography. The level of mRNA for MMP-2 was lower ($P <$

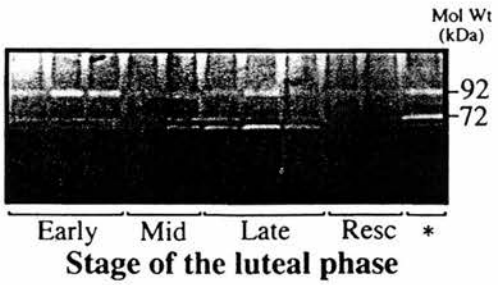

FIG. 1. Representative gelatin zymogram of human corpora lutea extracts from the early (LH+1 to LH+5), mid (LH+6 to LH+10), and late (LH+11 to LH+14) luteal phase and after luteal rescue by hCG (hCGx6 to hCGx8). The extract marked with an *asterisk* is taken from a corpus luteum that was rescued with hCG for 8 days and then collected 3 days after the final exposure to hCG. The bands are bright against a dark background, and the molecular size of each band in kilodaltons is indicated on the *left*.

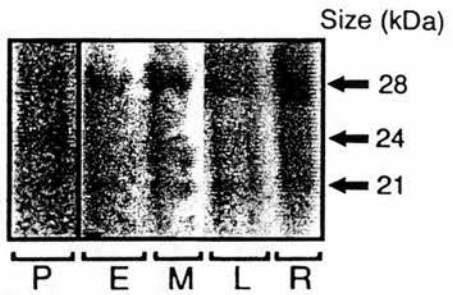

FIG. 2. Representative reverse zymogram of protein extracts from human placenta (P) and corpora lutea collected in the early (E; LH+1 to LH+5), mid (M; LH+6 to LH+10), and late (L; LH+11 to LH+14) luteal phase and after luteal rescue (R) with exogenous hCG (hCGx6 to hCGx8) *in vivo*. The bands are seen as dark against a lighter background, and the molecular size of each band in kilodaltons is indicated on the *left*.

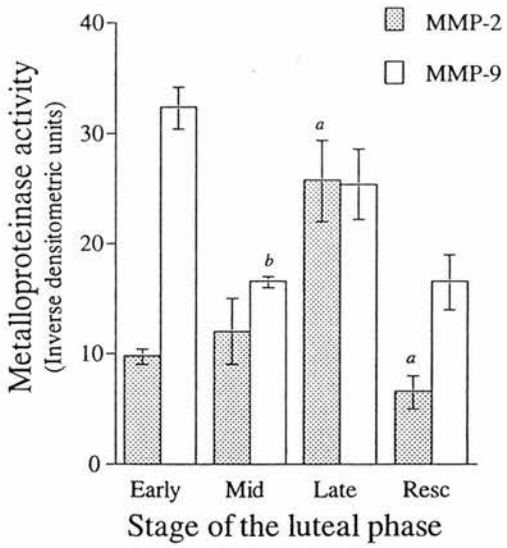

FIG. 3. Activities of MMP-2 and MMP-9 in human corpora lutea. The inverse intensity of the bands for MMP-9 (92-kDa) and the active form of MMP-2 (66-kDa) on gelatin zymography in the early (LH+1 to LH+5), mid (LH+6 to LH+10), and late (LH+11 to LH+14) luteal phase and after luteal rescue with hCG (hCGx6 to hCGx8). Values are the mean ± SD (n = 3/group). Differences ($P < 0.05$) in mean activities are shown (a, by *t* test; b, by ANOVA).

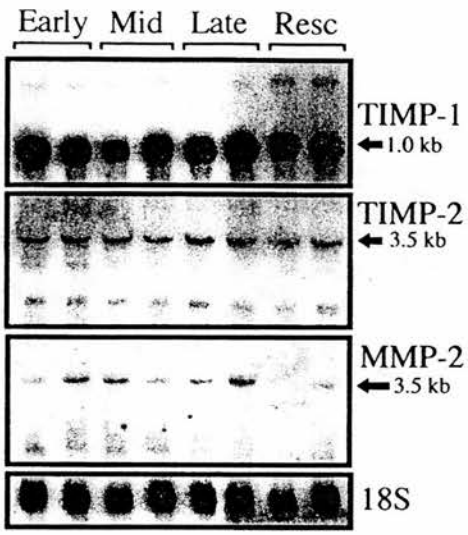

FIG. 4. Representative Northern blot for TIMP-1, TIMP-2, and MMP-2 in human corpora lutea in the early (LH+1 to LH+5), mid (LH+6 to LH+10), and late (LH+11 to LH+14) luteal phase and after luteal rescue with hCG (hCGx6 to hCGx8). Specific hybridization bands are dark against a lighter background. The approximate sizes in kilobases of the bands are indicated, and the 18S RNA bands are shown to demonstrate equal RNA loading.

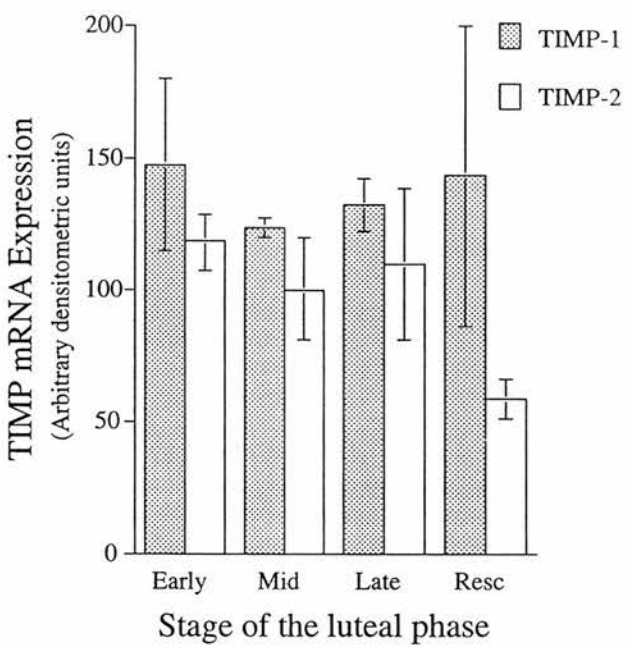

FIG. 5. Expression of TIMP-1 and TIMP-2 in the human corpus luteum. The intensities of TIMP-1 and TIMP-2 mRNAs, corrected for 18S intensity, in the early (LH+1 to LH+5), mid (LH+6 to LH+10), and late (LH+11 to LH+14) luteal phase and after luteal rescue with exogenous hCG (hCGx6 to hCGx8) *in vivo* are shown. Values are the mean ± SD (n = 3/group). There were no significant differences in the level of expression throughout the luteal phase (by ANOVA) or after luteal rescue (by *t* test).

0.05) in rescued corpora lutea than that in the late luteal phase in the absence of hCG (Fig. 6). There were no differences in MMP-1 expression throughout the luteal phase or after luteal rescue with exogenous hCG (Fig. 6).

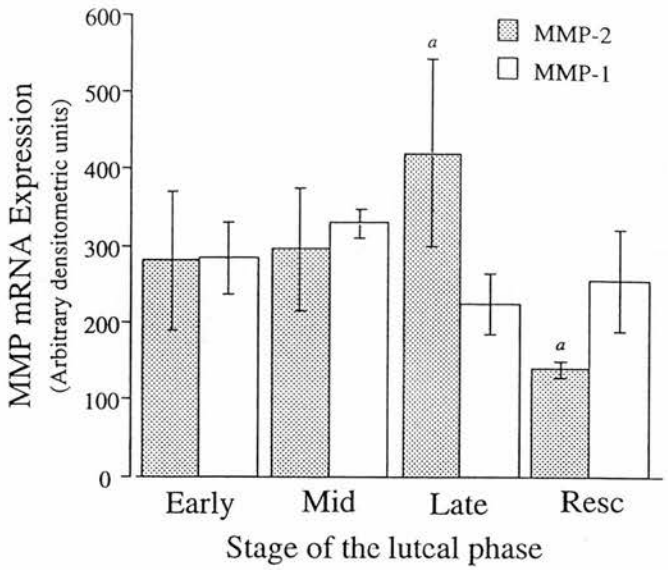

FIG. 6. Expression of MMP-1 and MMP-2 mRNA in the human corpus luteum. The intensities of the major MMP-1 and MMP-2 mRNA bands, corrected for 18S intensity, in the early (LH+1 to LH+5), mid (LH+6 to LH+10), and late (LH+11 to LH+14) luteal phase and after luteal rescue with exogenous hCG (hCGx6 to hCGx8) *in vivo* are shown. Values are the mean ± SD (n = 3/group). Significant differences are shown (a, $P < 0.05$, by ANOVA).

Localization of metalloproteinases and their tissue inhibitors

mRNA for TIMP-1, TIMP-2, MMP-1, MMP-2, and MMP-9 were localized in human corpora lutea by isotopic *in situ* hybridization. Each of these mRNA species had a specific pattern of localization that persisted throughout the normal luteal phase and after luteal rescue with exogenous hCG. In agreement with our previous findings, TIMP-1 was highly expressed in the granulosa-lutein cells of the corpus luteum (Fig. 7, a and b) (16). In contrast, TIMP-2 was localized to different regions of the corpus luteum. TIMP-2 was expressed at the periphery of the granulosa-lutein cells (Fig. 7c). Comparison with serial sections immunostained for 17α-hydroxylase to identify the thecal-lutein cells showed that TIMP-2 was expressed by the thecal-lutein cells (Fig. 7d). In addition, TIMP-2 was expressed in the fibrous connective tissue surrounding the steroidogenic cells (Fig. 7c).

MMP-1 was heavily expressed by the cells of the stroma and connective tissue of the corpus luteum (Fig. 8a). Only very low levels of expression were seen in the area of the gland containing steroidogenic cells (Fig. 8, a and b). MMP-2 was localized to the thecal-lutein cells and surrounding connective tissue (Fig. 8c). MMP-2 could also be detected around the vasculature and occasionally in cells within blood vessels. Little MMP-2 expression could be detected in the granulosa-lutein cell population. The distribution of MMP-9 was different, as expression was localized to individual cells within the gland (Fig. 8d). Although these cells were concentrated along the thecal-lutein cell layer and vascular connective tissue, some cells within the granulosa-lutein compartment were also found to express MMP-9 (Fig. 8d). This pattern of expression of MMPs and TIMPs was consistent and was seen in all serial sections examined (Fig. 9).

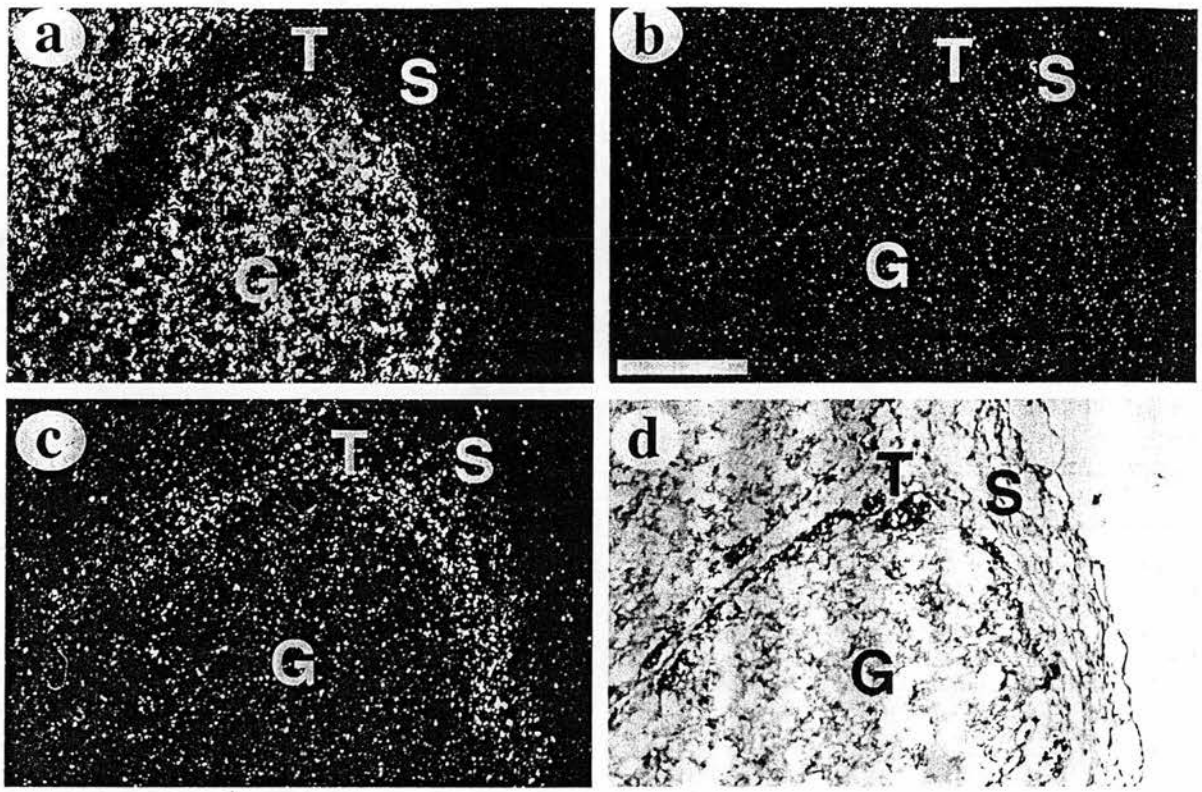

FIG. 7. Localization of TIMP-1 and TIMP-2 mRNA in the human corpus luteum. a, Darkfield image of TIMP-1 *in situ* hybridization in the early luteal corpus luteum, showing expression in the granulosa-lutein cells; b, negative control of a, showing few silver grains with no specific distribution; c, serial section of a, showing darkfield image of TIMP-2 *in situ* hybridization; expression of TIMP-2 is in a different cellular compartment from that of TIMP-1; d, serial section of c immunostained for 17α-hydroxylase to localize the thecal-lutein cells. G, Granulosa-lutein cells; T, thecal-lutein cells; S, connective tissue stroma. *Scale bar* = 100 μm.

Discussion

This study demonstrates the expression and localization of the MMPs and their specific tissue inhibitors in the primate corpus luteum. TIMP-1 has already been described as a major product of the corpus luteum of several species (12–16). We have now found that TIMP-2 is also expressed by the human corpus luteum. This agrees with the observation of Smith *et al.*, who described TIMP-2 expression in ovine (17) and bovine (18) follicles and corpora lutea. In contrast to TIMP-1 and TIMP-2, TIMP-3 is a not thought to be a secreted molecule, but, rather, is a component of the ECM (4, 32). Although we detected TIMP-3 mRNA in the human placenta, where it has previously been described (26), we found little expression in the corpus luteum. Similarly, Uria *et al.* did not detect TIMP-3 expression in the human ovary (33). However, TIMP-3 mRNA has been reported in the ovary of the pseudopregnant rat (11). This may reflect the different time periods examined, or it may be a species difference. In the human, it seems that TIMP-1 and TIMP-2 are the major luteal TIMPs.

In the small numbers we analyzed, the expression of TIMP-2 did not change during the functional luteal phase or after luteal rescue with exogenous hCG. TIMP-2 expression was found to change during the luteal phase in ovine corpora lutea (17). Smith *et al.* reported that TIMP-2 expression was maximal in the early luteal phase and significantly lower in the late luteal phase (17). In the cow, TIMP-2 expression was reported to increase significantly from the early to the mid-luteal phase (18), and expression was increased after PG-induced luteolysis (13). Further evidence of a species difference is that the primary TIMP-2 transcript size in the sheep corpus luteum is 1.0 kb (17), whereas in the human corpus luteum and other tissues (30) the size is 3.5 kb. In the human corpus luteum, control of tissue remodeling during the functional luteal phase does not appear to be related to alterations in the levels of expression of TIMPs.

Collagenase (MMP-1) and gelatinases A and B (MMP-2 and MMP-9) are expressed in the human corpus luteum. MMP-2 and MMP-9 have previously been detected by zymography in homogenates of rat ovaries (26), bovine corpus luteum (34), and luteinized human granulosa cells (35, 36). MMP-1, MMP-2, and MMP-9 mRNAs have been described in the pseudopregnant rat ovary (37). Collagen and other components of the ECM are an integral part of the structure of the corpus luteum (3, 38). The human corpus luteum expresses enzymes with the capacity to proteolytically break down these components of the ECM.

The expression and activity of MMPs in the corpus luteum changed during the luteal phase. MMP-2 expression and activity were maximal in the late luteal corpus luteum. This is consistent with a role in tissue remodeling associated with luteolysis. In the rat, PRL-induced structural luteolysis was associated with the activity of metalloproteinase enzymes, particularly MMP-2 (26). Interestingly, Aston *et al.* recently

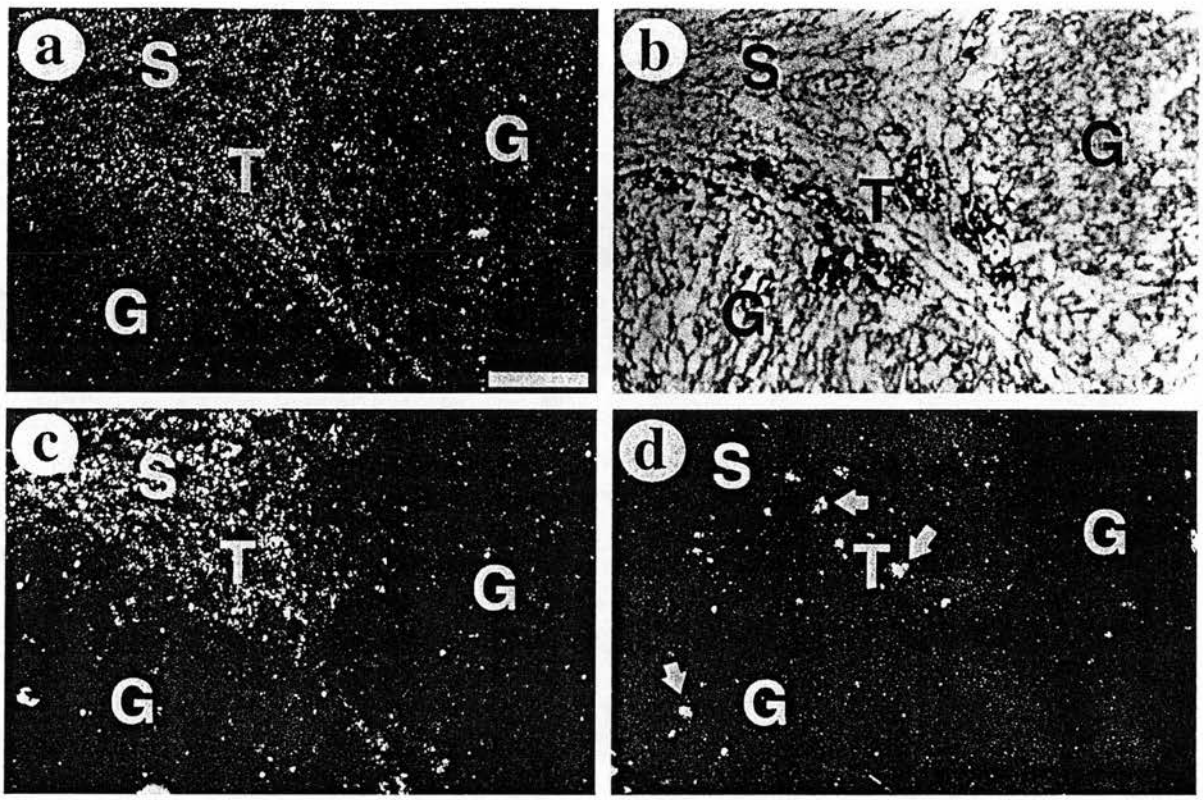

FIG. 8. Localization of mRNA for the major MMPs in the human corpus luteum. a, Darkfield image of *in situ* hybridization for MMP-1 in the midluteal phase corpus luteum, showing expression in the connective tissue stroma with minimal expression in the granulosa-lutein cell layer; b, serial section of a immunostained for 17α-hydroxylase to localize the thecal-lutein cells; c, darkfield *in situ* hybridization showing the localization of MMP-2 mRNA; d, darkfield *in situ* hybridization showing the localization of MMP-9 mRNA in the same corpus luteum. G, Granulosa-lutein cells; T, thecal-lutein cells; S, connective tissue stroma. *Scale bar* = 100 μm.

reported that MMP-2 activity increased with length of time of culture of luteinized granulosa cells (36). The major MMP secreted from ovine luteal explants was MMP-2 (39). Expression of MMP-2 in the corpus luteum may be associated with the tissue remodeling at the time of luteolysis.

In contrast, high levels of MMP-9 activity were also detected in the early luteal phase. It is possible that MMP-9 is involved in the extensive tissue remodeling that occurs during the formation of the corpus luteum from the ruptured follicle (3). A role of MMP-9 in the formation of the corpus luteum is supported by the finding that it is the primary metalloproteinase detected in follicle explants (39). In addition, MMP-9 is the major MMP secreted into the culture medium of luteinized bovine (38) and human granulosa cells (35, 36). Dispersed luteal cells from 4-day-old bovine corpora lutea had both MMP-2 and MMP-9 activities, but MMP-9 activity decreased with duration of culture (34), and MMP-9 was seen in the medium of cultured human granulosa cells only during the first 2 days of culture (35). This provides preliminary evidence that MMP-9 may have a role in ovulation and the tissue remodeling associated with the formation of the corpus luteum.

Compared to that during the late luteal phase, exposure of the corpus luteum to hCG during luteal rescue was associated with reduced expression and activity of MMP-2. This is clearly different from the process of ovulation, when LH/hCG stimulates an increase in MMP-1 and MMP-2 expression (5, 40, 41). Follicular levels of MMP-2 increase between the LH surge and ovulation (39). In cultures of luteinized granulosa cells, hCG also was shown to reduce the expression of MMP-2 and MMP-9 (36, 42). Human granulosa cells cultured on a thin layer of ECM are lost from culture in the absence of gonadotropin (36). These cells are released from culture via an active process suppressed by hCG (43). One of the effects of hCG during maternal recognition of pregnancy appears to be the inhibition of metalloproteinase expression.

MMP-1 and MMP-2 had similar cellular localizations in the human corpus luteum. They were expressed in the connective tissue stroma, the vascular pedicles, and the thecal-lutein cell layer. Fibroblasts and endothelial cells are sources of MMPs (44), and they are likely to express MMP-1 and MMP-2 in the corpus luteum. In the endometrium (7) and in ovarian cancers (8), cells of the stroma also have been shown to express these enzymes. Although the expression of MMP-2 was maximal in the late luteal phase, its localization in the corpus luteum was not affected. This suggests that the source of MMP during luteolysis is the periphery of the gland. In contrast, MMP-9 mRNA was localized to single cells in steroidogenic and nonsteroidogenic cell layers. The identity of these cells is uncertain, but they are probably white blood cells. Polymorphonuclear leukocytes express MMP-9 (45), and we found that expression was often associated with blood vessels. Cells of the immune system, including macrophages, are also constituents of the human

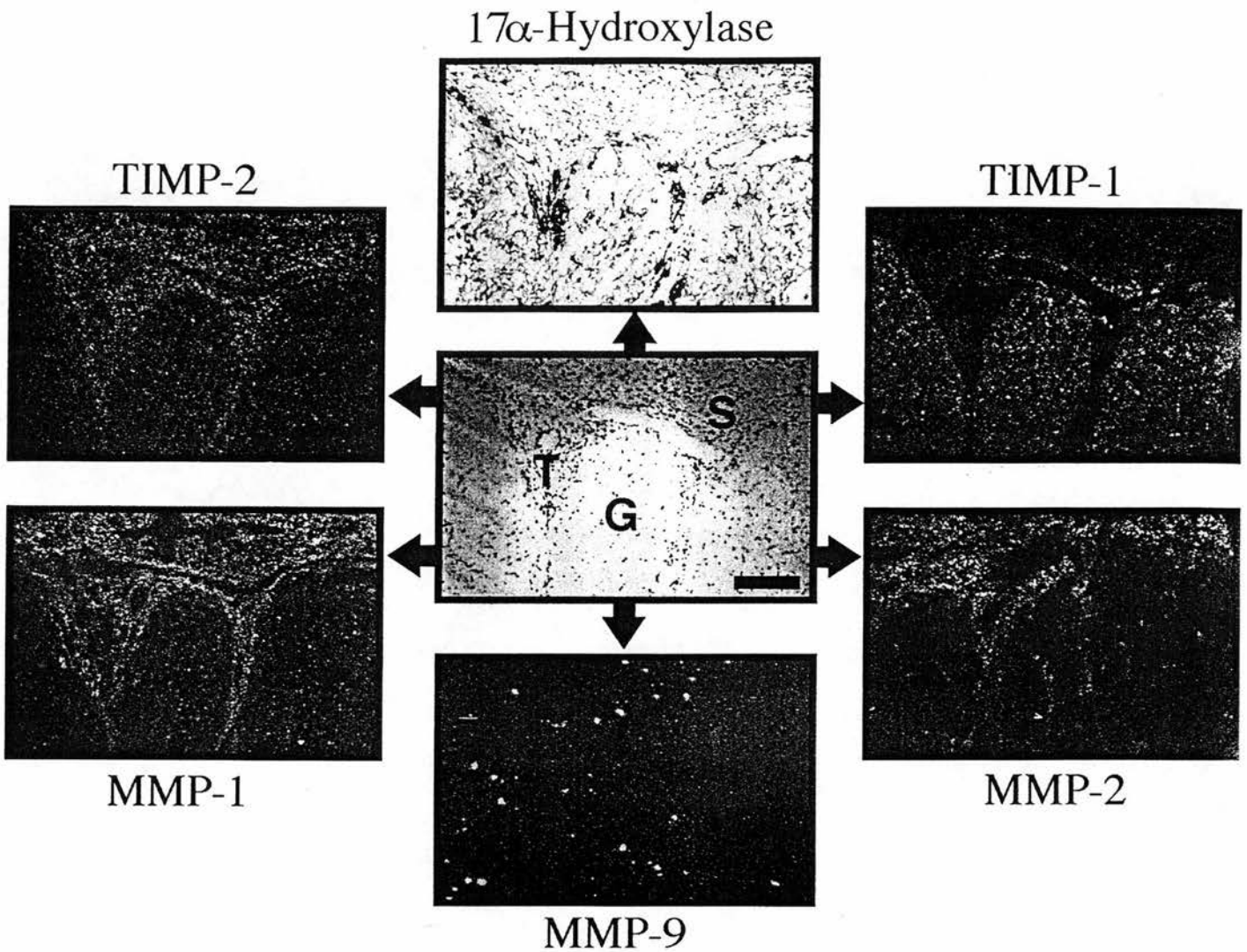

FIG. 9. Relationship between the localization of the major MMPs and TIMPs in the late luteal corpus luteum. A composition of serial sections after *in situ* hybridization for MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 and immunohistochemistry for 17α-hydroxylase arranged around the lightfield section of the corpus luteum showing the pattern of expression of MMPs and TIMPs. G, Granulosa-lutein cells; T, thecal-lutein cells; S, connective tissue stroma. *Scale bar* = 200 μm.

corpus luteum (46) and may be a source, or stimulator, of MMP expression.

It is unclear whether MMPs are expressed by the granulosa-lutein cells of the corpus luteum. Few grains were localized to this cell layer, and when present, they were in isolated individual cells. This finding is contrary to reports using cultures of luteinized granulosa cells (35, 36). *In vitro* MMP-9 expression falls with continuing culture. This has led some researchers to suggest that MMP-9 activity is related to leukocytes that accompany the granulosa cells in the first few days of culture (35). However, it is thought that bovine and human granulosa cells and bovine luteal cell dispersates in culture secrete MMP-2 (34, 35, 38). Although it is possible that MMP-2 activity in these cultures results from white cell or thecal contamination, it is likely that granulosa-lutein cells have the potential to express MMPs and are induced to do so in culture. However, it is clear that the main site of MMP-2 expression in the corpus luteum is not the granulosa-lutein cells.

TIMP-1 and TIMP-2 have different cellular localizations in the corpus luteum. TIMP-2 was localized to the thecal-lutein cells and the surrounding connective tissue stroma. Smith *et al.* found TIMP-2 in the theca of the ovine follicle (17). This is consistent with the primary localization of TIMP-2 in the follicle being maintained in the mature corpus luteum. The localization of TIMP-2 was similar to those of MMP-1 and MMP-2. This suggests that TIMP-2 may have a role in the local regulation of these enzymes in the corpus luteum. Indeed, it has been suggested that TIMP-2 displays a preference for MMP-2 (47). However, as we have previously reported (16), the localization of TIMP-1 is different. It is possible that TIMP-1 has other roles in addition to inhibition of metalloproteinases in the corpus luteum (17, 48, 49). However, the lack of significant ovarian disturbance in mice without a functional TIMP-1 gene (50) means that the role of high TIMP-1 expression in granulosa-lutein cells is not clear.

It was not clear how MMPs could function in the corpus luteum, which expresses large amounts of the specific in-

hibitor TIMP-1 (13, 16). We have shown that MMPs are expressed in different areas of the corpus luteum than TIMP-1. In addition, where MMPs were expressed in the granulosa-lutein cellular layer, expression was in foci of individual cells. The localization of MMPs seems to be a key factor in their activity in the corpus luteum.

In conclusion, the expression of MMP-2 in the late luteal phase may indicate a role for this enzyme in the tissue remodeling associated with luteolysis. One function of hCG during luteal rescue is to prevent this increase in MMP expression. As TIMP-1 and TIMP-2 change little, it is likely that control of MMP activity in the corpus luteum involves changing MMP, rather than TIMP, expression. MMPs are localized in different areas than TIMP-1, and where they are expressed in the same area, they are expressed in foci. This may explain how MMPs can function in the background of large amounts of TIMP-1.

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References

1. **Behrman HR, Endo T, Aten RF, Musicki B.** 1993 Corpus luteum function and regression. Reprod Med Rev. 2:153–180.
2. **Ford SP, Reynolds LP, Magness RR.** 1982 Blood flow to the uterine and ovarian vascular beds of gilts during the estrous cycle or early pregnancy. Biol Reprod. 27:878–885.
3. **Luck MR, Zhao Y.** 1995 Structural remodelling of reproductive tissues. J Endocrinol. 146:191–195.
4. **Salamonsen LA.** 1996 Matrix metalloproteinases and their tissue inhibitors in endocrinology. Trends Endocrinol Metab. 7:28–34.
5. **Hulboy DL, Rudolph LA, Matrisian LM.** 1997 Matrix metalloproteinases as mediators of reproductive function. Mol Hum Reprod. 3:27–45.
6. **Reich R, Tsafriri A, Mechanic GL.** 1985 Involvement of collagenolysis in ovulation in the rat. Endocrinology. 116:522–527.
7. **Hampton AL, Salamonsen LA.** 1994 Expression of messenger ribonucleic acid encoding matrix metalloproteinases and their tissue inhibitors is related to menstruation. J Endocrinol 141:R1–R3.
8. **Naylor MS, Stamp GW, Davies BD, Balkwill FR.** 1994 Expression and activity of MMPs and their regulators in ovarian cancer. Int J Cancer. 58:50–56.
9. **Matrisian LM.** 1990 Metalloproteinases and their inhibitors in matrix remodelling. Trends Genet. 6:121–125.
10. **Birkedal-Hansen H.** 1995 Proteolytic remodeling of the extracellular matrix. Curr Opin Cell Biol. 7:728–735.
11. **Nothnick WB, Edwards DR, Leco KJ, Curry Jr TE.** 1995 Expression and activity of ovarian tissue inhibitors of metalloproteinases during pseudopregnancy in the rat. Biol Reprod. 53:684–691.
12. **Smith GW, Goetz TL, Anthony RV, Smith MF.** 1994 Molecular cloning of an ovine ovarian tissue inhibitor of metalloproteinases: ontogeny of messenger ribonucleic acid expression and *in situ* localization within preovulatory follicles and luteal tissue. Endocrinology. 134:344–352.
13. **Juengel JL, Smith GW, Smith MF, Youngquist RS, Garverick HA.** 1994 Pattern of protein production by bovine corpora lutea during luteolysis and characterization of expression of two major secretory products of the regressing corpora lutea. J Reprod Fertil. 100:515–520.
14. **Smith MF, Kemper CN, Smith GW, Goetz TL, Jarrell VL.** 1994 Production of tissue inhibitor of metalloproteinases-1 by porcine follicular and luteal cells. J Anim Sci. 72:1004–1012.
15. **Duncan WC, Illingworth PJ, Fraser HM.** 1996 Expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the primate ovary during induced luteal regression. J Endocrinol. 151:203–213.
16. **Duncan WC, McNeilly AS, Illingworth PJ.** 1996 Expression of tissue inhibitor of metalloproteinases-1 in the human corpus luteum after luteal rescue. J Endocrinol. 148:59–67.
17. **Smith GW, McCrone S, Petersen SL, Smith MF.** 1995 Expression of messenger ribonucleic acid encoding tissue inhibitor of metalloproteinases-2 within ovine follicles and corpora lutea. Endocrinology. 136:570–576.
18. **Smith GW, Juengel JL, McIntush EW, Youngquist RS, Garverick HA, Smith MF.** 1996 Ontogenies of messenger RNA encoding tissue inhibitor of metalloproteinases 1 and 2 within bovine periovulatory follicles and luteal tissue. Dom Anim Endocrinol. 13:151–160.
19. **Djahanbakhch O, McNeilly AS, Hobson BM, Templeton AA.** 1981 A rapid luteinizing hormone radioimmunoassay for the prediction of ovulation. Br J Obstet Gynaecol. 88:1016–1020.
20. **Illingworth PJ, Reddi K, Smith K, Baird DT.** 1990 Pharmacologic 'rescue' of the corpus luteum results in increased inhibin production. Clin Endocrinol (Oxf). 33:323–332.
21. **Li TC, Rogers AW, Dockery P, Lenton EA, Cooke ID.** 1988 A new method of histologic dating of human endometrium in the luteal phase. Fertil Steril. 50:52–60.
22. **Djahanbakhch O, Swanston IA, Corrie JET, McNeilly AS.** 1981 Prediction of ovulation by progesterone. Lancet. 2:1164–1165.
23. **Bradford MM.** 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248–254.
24. **Chomczynski P, Sacchi N.** 1987 Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162:156–159.
25. **Brooks J, Crow WJ, McNeilly JR, McNeilly AS.** 1992 Relationship between gonadotropin subunit gene-expression, gonadotropin-releasing hormone receptor content and pituitary and plasma gonadotropin concentrations during the rebound release of FSH after treatment of ewes with bovine follicular-fluid during the luteal phase of the cycle. J Mol Endocrinol. 8:109–118.
26. **Endo T, Aten RF, Wang F, Behrman HR.** 1993 Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luterolysis. Endocrinology. 133:690–698.
27. **Hampton AL, Butt AR, Riley SC, Salamonsen LA.** 1995 Tissue inhibitors of metalloproteinases in endometrium of overiectomized steroid-treated ewes and during the estrous cycle and early pregnancy. Biol Reprod. 53:302–311.
28. **Higuchi T, Kanzaki H, Nakayama H, et al.** 1995 Induction of tissue inhibitor of metalloproteinase 3 gene expression during *in vitro* decidualization of human endometrial stromal cells. Endocrinology. 136:4973–4981.
29. **Rapp G, Freudstein J, Klaudiny J, et al.** 1990 Characterisation of three abundant mRNAs from human ovarian granulosa cells. DNA Cell Biol. 9:479–485.
30. **Stetler-Stevenson WG, Brown PD, Onisto M, Levy AT, Liotta LA.** 1990 Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell line and human tumor tissues. J Biol Chem. 265:13933–13938.
31. **Hoeben E, Vanaelst I, Swinnen JV, Openakker G, Verhoeven G.** 1996 Gelatinase-A secretion and its control in peritubular and sertoli-cell cultures: effects of hormones, 2nd-messengers and inducers of cytokine production. Mol Cell Endocrinol. 118:37–46.
32. **Leco KJ, Khokha R, Pavloff N, Hawkes SP, Edwards DR.** 1994 Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. J Biol Chem. 269:9352–9360.
33. **Uria JA, Ferrando AA, Velasco G, Freije JMP, Lopez-Otin C.** 1994 Structure and expression in breast tumors of human TIMP-3, a new member of the metalloproteinase inhibitor family. Cancer Res. 54:2091–2094.
34. **Tsang PCW, Poff JP, Boulton EP, Condon WA.** 1995 Four-day-old bovine corpus luteum: progesterone production and identification of matrix metalloproteinase activity *in vitro*. Biol Reprod. 53:1160–1168.
35. **Puistola U, Westerlund A, Kauppila A, Turpeenniemi-Hujanen T.** 1995 Regulation of 72-kd type IV collagenase-matrix metalloproteinase-2 by estradiol and gonadotrophin-releasing hormone agonist in human granulosa-lutein cells. Fertil Steril. 64:81–87.
36. **Aston KE, Stamouli A, Thomas EJ, et al.** 1996 Effect of gonadotrophin on cell and matrix retention and expression of metalloproteinases and their inhibitor in cultured human granulosa cells modelling corpus luteum function. Mol Hum Reprod. 2:26–30.
37. **Nothnick WB, Keeble SC, Curry Jr TE.** 1996 Collagenase, gelatinase, and proteoglycanase messenger ribonucleic acid expression and activity during luteal development, maintenance, and regression in the pseudopregnant rat ovary. Biol Reprod. 54:616–624.
38. **Zhao Y, Luck MR.** 1996 Bovine granulosa cells express extracellular matrix proteins and their regulators during luteinization in culture. Reprod Fertil Dev. 8:259–266.
39. **Russell DL, Salamonsen LA, Findlay JK.** 1995 Immunization against the N-terminal peptide of the inhibin α43-subunit (αN) disrupts tissue remodeling and the increase in matrix metalloproteinase-2 during ovulation. Endocrinology. 136:2657–3664.
40. **Tadakuma H, Okamora H, Kitaoka M, Iyama K, Usuku G.** 1993 Association of immunolocalization of matrix metalloproteinase 1 with ovulation in hCG-treated rat ovary. J Reprod Fertil. 98:1869–1875.
41. **Curry TE, Mann JS, Huang MH, Keeble SC.** 1992 Gelatinase and proteoglycanase activity during the periovulatory period in the rat. Biol Reprod. 46:256–264.
42. **Stamouli A, O'Sullivan MJB, Frankel S, Thomas EJ, Richardson MC.** 1996

Suppression of matrix metalloproteinase production by hCG in cultures of human luteinized granulosa cells as a model for gonadotrophin-induced luteal rescue. J Reprod Fertil. 105:235–239.

43. **Aston KE, O'Sullivan MJB, Thomas EJ, Richardson MC.** 1996 Effect of human chorionic gonadotrophin on the detachment of human granulosa cells from extracellular matrix layered onto glass or plastic. Hum Reprod. 11:336–340.
44. **Woessner Jr JF.** 1991 matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 5:2145–2154.
45. **Murphy G, Ward R, Hembry RM, Raynolds JJ, Kühn K, Tryggvason K.** 1989 Characterization of gelatinase from pig polymorphonuclear leucocytes. Biochem J. 258:463–472.
46. **Brannstrom M, Pascoe V, Norman RJ, McClure N.** 1994 Localization of leukocyte subsets in the follicle wall and in the corpus-luteum throughout the human menstrual-cycle. Fertil Steril. 61:488–495.
47. **Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S, He CS.** 1989 Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteinases designated TIMP-2. Proc Natl Acad Sci USA. 86:8207–8211.
48. **Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K.** 1992 Growth promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. FEBS Lett. 298:29–32.
49. **Boujrad N, Ogwuegbu SO, Garnier M, Lee C-H, Martin BM, Papadopoulos V.** 1995 Identification of a stimulator of steroid hormone synthesis isolated from testis. Science. 268:1609–1612.
50. **Nothnick WB, Soloway P, Curry Jr TE.** 1997 Assessment of the role of tissue inhibitor of metalloproteinase-1 (TIMP-1) during the periovulatory period in female mice lacking a functional TIMP-1 gene. Biol Reprod. 56:1181–1188.

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The human corpus luteum: reduction in macrophages during simulated maternal recognition of pregnancy

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It has been shown that immune cells, particularly macrophages, accumulate in the corpus luteum during luteolysis. This study aimed to investigate the effect of maternal recognition of pregnancy on the localization and numbers of macrophages in the human corpus luteum. Corpora lutea (n = 12) were obtained from normally cycling women at the time of hysterectomy and were dated on the basis of serial urinary luteinizing hormone (LH) estimation. In addition, corpora lutea (n = 4) were collected from women who had received daily doubling doses of human chorionic gonadotrophin (HCG) to mimic the hormonal changes of early pregnancy. Macrophages were localized by immunohistochemistry using an anti-CD68 antibody. Steroidogenic cells, steroidogenic cells of thecal origin and endothelial cells were identified on serial sections by immunohistochemistry for 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase and von Willebrand factor, respectively. The luteal cells capable of responding directly to HCG were identified by isotopic in-situ hybridization for messenger RNA encoding LH/HCG receptors. Macrophages were localized primarily to the vascular connective tissue and theca–lutein areas of the corpus luteum, although some were found in the granulosa–lutein cell layer. Macrophage numbers increased throughout the luteal phase to a maximum in the late-luteal phase ($P < 0.05$). Luteal 'rescue' with HCG was associated with a marked reduction in the numbers of tissue macrophages when compared with those of the late-luteal phase ($P < 0.001$). One of the effects of HCG during maternal recognition of pregnancy is to prevent the normal influx of macrophages into the corpus luteum. As LH/HCG receptors localized to the steroidogenic cells, this implies a fundamental role for steroidogenic cell products in the control of macrophage influx into the human corpus luteum.

Key words: corpus luteum/macrophages/human chorionic gonadotrophin/pregnancy/luteolysis

Introduction

In a non-conception cycle, the primate corpus luteum undergoes luteolysis with a loss of functional and structural integrity. The molecular events involved in luteolysis and how they are prevented by exposure to human chorionic gonadotrophin (HCG) during maternal recognition of pregnancy remain unclear (Behrman *et al.*, 1993). One feature of luteolysis, however, is the marked accumulation of immune cells in the corpus luteum. This increase in the number of immune cells has been reported in a variety of species, including rodents (Brännström *et al.*, 1994a), rabbits (Naftalin *et al.*, 1997), ruminants (Murdoch, 1987; Lei *et al.*, 1991), and women (Wang *et al.*, 1992a; Best *et al.*, 1996; Takaya *et al.*, 1997). As these species use disparate mechanisms to control their corpora lutea (Auletta and Flint, 1988), this common increase implies a fundamental role for immune cells, or their cytokine products (Brännström and Norman, 1993), in the luteolytic process.

The main immune cell to be localized in the human corpus luteum during luteolysis is the macrophage (Wang *et al.*, 1992a; Brännström *et al.*, 1994b; Best *et al.*, 1996). However, macrophage products have been shown to have both positive and negative effects on progesterone secretion. Macrophage products have been reported to have pro-steroidogenic effects in cell culture (Kirsch *et al.*, 1981, 1983; Halme *et al.*, 1985). In addition, in the early stages of luteal function, macrophages are thought to have primarily luteotrophic effects (Brännström and Norman, 1993). Other macrophage products, however, such as tumour necrosis factor α (TNFα), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), reactive oxygen species and nitric oxide, have been shown to have negative effects on steroidogenesis (Benyo and Pate, 1992; Grusenmeyer and Pate, 1992; Van Voorhis *et al.*, 1994; Vega *et al.*, 1995; Kato *et al.*, 1997). Whereas macrophages are likely to be involved in the phagocytic clearance of cell debris (Paavola, 1979; Takaya *et al.*, 1997) after functional luteolysis, it is still not clear what role tissue macrophages have in the functional corpus luteum.

We postulated that the primary role of macrophages in the human corpus luteum was luteolytic, rather than luteotrophic, and that macrophage numbers would not increase in the 'rescued' corpus luteum of early pregnancy. To test this hypothesis, we investigated the number of macrophages in the human corpus luteum throughout the functional luteal phase by immunohistochemistry. We compared the numbers of macrophages in corpora lutea in the late-luteal phase, where progesterone output is falling, with corpora lutea 'rescued' with exogenous HCG, where progesterone output is increasing. We also postulated that any increase in macrophages within the corpus luteum was by de-novo influx, rather than by local changes in cellular localization. To test this hypothesis, we identified the structural architecture of the corpus luteum by immunohistochemistry and compared the localization of

macrophages throughout the luteal phase and after luteal 'rescue' with exogenous HCG. In the final part of the study, we investigated the site of action of HCG on macrophages during luteal 'rescue' by localizing luteinizing hormone (LH)/HCG receptors in the corpus luteum by isotopic in-situ hybridization.

Materials and methods

Source of reagents

All reagents were obtained from Sigma Chemical (Poole, Dorset, UK), unless otherwise stated. The mouse monoclonal antibodies to human CD68 (PG-M1) and von Willebrand factor were obtained commercially (Dako Ltd, High Wycombe, Bucks, UK). The polyclonal rabbit antibody to human 17α-hydroxylase was kindly provided by Prof. M.R. Waterman (Vanderbilt University, Nashville, TN, USA). The polyclonal rabbit antibody to human placental type I 3β-hydroxysteroid dehydrogenase (3β-HSD) was kindly provided by Prof. Van Luu-The (CHUL Research Centre, Quebec, Canada). A 1.5-kb cDNA construct, corresponding to nucleotide 542 to the last nucleotide of the open reading frame (2124), of the human LH receptor in pBluescript (Stratagene, Cambridge, Cambs, UK) was kindly provided by Dr M.Atger of the Faculté de Médècine de Bicêtre, Université Paris-Sud, Le Kremlin-Bicêtre, France.

Collection of tissue

Corpora lutea were enucleated at the time of hysterectomy in 16 women undergoing surgery for benign conditions, typically dysmenorrhoea, uterine fibroids or menorrhagia. All women were healthy, aged from 32–45 years with regular menstrual cycles and had not received any form of hormonal treatment for at least 3 months prior to taking part in the study. The corpora lutea were dated on the basis of serial urinary LH measurements on samples collected daily prior to operation (Djahanbakhch *et al.*, 1981a). On this basis, four corpora lutea were classified as early- (LH+1 to LH+5), four as mid- (LH+6 to LH+10) and four as late- (LH+11 to LH+14) luteal. In addition, four women received daily intramuscular injections of HCG (Profasi: Serono Laboratories, Welwyn Garden City, UK) from LH+7 in daily doubling doses, starting at 125 IU, for 5 to 8 days until surgery. This regimen has been shown to reproduce the hormonal changes of early pregnancy (Illingworth *et al.*, 1990).

The whole corpus luteum was enucleated from the ovary by blunt dissection and the ovary oversewn as previously described (Duncan *et al.*, 1996a,b). The tissue was divided immediately into radial blocks in order to ensure that the whole thickness of the gland was represented in any piece. One piece was fixed in 4% paraformaldehyde for 24 h and embedded in paraffin wax for subsequent immunohistochemistry and another piece was frozen in embedding medium (Tissue-Tek OCT compound, Miles Inc., Elkhart, IN, USA) and stored at –70°C. Frozen sections (5 μm) were cut from this block on to poly-L-lysine (50 μg/l)-coated slides and stored at –70°C until use. In each case an endometrial biopsy was also fixed in paraformaldehyde and processed into paraffin wax for luteal-phase dating by tissue morphometry (Li *et al.*, 1988). Plasma was taken before surgery and progesterone concentration was measured by a standard radioimmunoassay (Djahanbakhch *et al.*, 1981b). This study was approved by the Reproductive Medicine Subcommittee of the South East Scotland Research Ethics Committee, and informed consent was obtained from all patients prior to tissue collection.

Immunohistochemistry

Five-micrometre paraffin wax sections were cut on to poly-L-lysine (50 μg/l)-coated slides, de-waxed and rehydrated. As preliminary experiments indicated that antigen retrieval using trypsinization was necessary for the detection of the CD68 antigen and von Willebrand factor, these sections were incubated in 0.1% (w/v) trypsin with 0.1% (w/v) calcium chloride, buffered to pH 7.4 with 0.25 M Tris–HCl, for 30 min at 37°C. The sections were then washed in 0.05 M Tris-buffered saline (TBS) pH 8. Endogenous peroxidase activity was blocked with 2% (v/v) hydrogen peroxide in 60% methanol for 30 min at room temperature. This tissue was then permeabilized with 0.1% Triton-X100 in TBS and rinsed in TBS prior to blocking with 20% (v/v) normal rabbit serum in TBS with 4% (w/v) bovine serum albumin for 20 min. Sections were incubated with the primary antibody, monoclonal mouse anti-human macrophage CD68 antigen diluted 1:50 in TBS, or the mouse anti-human von Willebrand factor diluted 1:25, for 1 h at room temperature (Rodger *et al.*, 1997). Mouse immunoglobulin G (IgG) (Vector Laboratories, Peterborough, Cambs, UK) at an equivalent antibody concentration was used as a negative control.

Antibody binding was indicated by an avidin–biotin horseradish peroxidase (Dako Ltd) reaction with a biotinylated rabbit anti-mouse (Dako Ltd) secondary antibody at a dilution of 1:100 in TBS. The reaction was developed with diamino-benzidine to give a stable brown end-product (Vector Laboratories). Sections were then washed in water, counter-stained with haematoxylin, dehydrated through graded alcohols and mounted with Pertex mounting medium (Cellpath, Hemel Hempstead, Herts, UK).

Steroidogenic cells were identified in serial sections by immunohistochemistry for 3β-HSD (Riley *et al.*, 1992). Here, polyclonal rabbit anti-human 3β-HSD was used in a dilution of 1:1000. Immunohistochemistry was performed as described above, without trypsinization, using normal goat serum (SAPU, Carluke, Lanarkshire, UK) to block non-specific binding, and specific binding was detected using biotinylated goat anti-rabbit immunoglobulins (Dako Ltd). Steroidogenic cells of thecal origin were identified in serial sections by immunohistochemistry for 17α-hydroxylase as described previously (Rodger *et al.*, 1995; Duncan *et al.*, 1996a). Briefly, the polyclonal rabbit anti-human 17α-hydroxylase antibody was used at a 1:750 dilution, normal goat serum was used to block non-specific binding, and biotinylated goat anti-rabbit immunoglobulins were used as the secondary antibody. Rabbit serum with an equivalent immunoglobulin concentration (Dako Ltd) was used as a negative control.

In-situ hybridization

Isotopic in-situ hybridization for LH/HCG receptors was performed using antisense and sense [^{35}S]-labelled riboprobes as described previously (Duncan *et al.*, 1996b). Briefly, the antisense probe, incorporating [^{35}S]-labelled UTP (Amersham International plc, Aylesbury, Bucks, UK), was generated from the plasmid vector linearized by HindIII (Promega, Southampton, Hants, UK) using T3 RNA polymerase (Promega). The [^{35}S]-labelled sense probe was used as a negative control. This was generated from the plasmid vector linearized by ECoR1 (Promega) using T7 RNA polymerase (Promega).

As preliminary experiments with fixed serial sections gave technically poor results, LH/HCG receptor mRNA was localized in frozen sections from the same corpora lutea. Frozen sections (5 μm) were fixed in 4% paraformaldehyde, rinsed and then acetylated in 0.25% (v/v) acetic anhydride (BDH Laboratory Supplies, Poole, Dorset, UK). After dehydrating through graded alcohols and drying under vacuum, 100 μl of hybridization buffer (50% deionized formamide, 10% dextran sulphate, 1×Denhardt's solution, 0.5 mg/ml yeast tRNA, 10 mM dithiothreitol, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8) containing 1×10^6 cpm radiolabelled riboprobe was added to each section, and the slides were incubated overnight at 55°C in a moist chamber.

The following day the slides were treated with RNase A (20 μg/ml), and washed in increasingly stringent conditions. The sections were then dehydrated through graded alcohols, allowed to dry and dipped in Kodak NTB-2 photographic emulsion (IBI Ltd, Cambridge, Cambs, UK). After incubation in the dark for 21 days, they were developed (Kodak D-19) and fixed (Kodak Unifix) at 15°C. The sections were then rinsed, counter-stained with haematoxylin and mounted. They were viewed under dark-field illumination, and the localization of the silver grains was determined by reference to the image viewed under light-field illumination.

Image analysis

The number of macrophages was counted by an observer blinded to the tissue identity, and this was repeated the following week to confirm the reproducibility of the results. Macrophage numbers in all sections during the repeat count were within 5% of the initial count. Macrophages were identified by intense brown staining on tissue sections. Only positive cells where the nuclei could be identified were counted in order to avoid counting tiny fragments of cells present in the tissue section. Sections were analysed using a stratified random sampling technique using a graticule lens. The stratified random sampling technique involved taking random fields from a grid of 24 fields arranged around a fixed, non-random, point (the centre of the section). At least five fields of each section were counted and the running mean was monitored to confirm adequate sampling. The granulosa–lutein cell layers, the theca–lutein cell layers and the surrounding stroma were identified by morphology and by comparison with serial sections immunostained for 3β-HSD and 17α-hydroxylase. In addition to the total number of macrophages, the number of macrophages in these layers was also recorded.

The number of macrophages at different stages of the luteal phase, and the number in the different cellular layers, were analysed by one-way analysis of variance with a 5% level of significance. Where significant differences were found to exist, pairwise comparisons using the Bonferroni/Dunn method were performed using commercial computer software (StatView 4.0; Abacus Concepts Inc., Berkeley, CA, USA).

Results

Plasma progesterone concentrations

The classification of the corpora lutea by serial urinary LH measurement agreed with the luteal-phase dating of endometrial biopsies using the method of Li *et al.* (1988). As reported previously (Duncan *et al.*, 1996b), the plasma progesterone concentrations were 36.36 ± 9.28 nmol/l in the early luteal samples, 40.35 ± 9.88 nmol/l in the mid-luteal samples and 18.80 ± 12.81 nmol/l in the late luteal samples. After luteal 'rescue' by exogenous HCG the plasma progesterone concentrations had increased to 52.75 ± 1.09 nmol/l.

Functional anatomy of the corpus luteum

Steroidogenic cells were identified in human corpora lutea by the immunolocalization of 3β-HSD (Figure 1a). Luteal cells of both thecal and granulosal origin express this enzyme. The theca–lutein cells were specifically identified by immunolocalization of 17α-hydroxylase (Figure 1b). These cells formed clearly distinct populations, located around the peripheral margin of the granulosa–lutein cells. Vascular endothelial cells were localized by immunohistochemistry for von Willebrand factor (Figure 1c). The theca–lutein cell layer had a rich blood

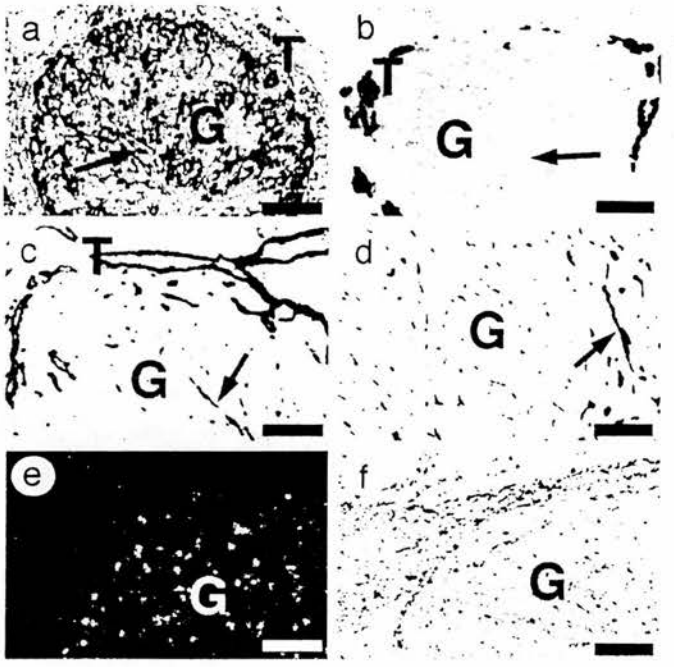

Figure 1. Functional anatomy of the human corpus luteum: (**a**) mid-luteal corpus luteum immunostained for 3β-hydroxysteroid dehydrogenase showing staining of the steroidogenic cells with no staining of the connective tissue core; (**b**) the same corpus luteum as (**a**) immunostained for 17α-hydroxylase showing staining in the theca–lutein cells; (**c**) the same corpus luteum as (**a**) immunostained for von Willebrand factor to demonstrate the endothelial cells; (**d**) a closer view of the granulosa–lutein cell layer of another mid-luteal corpus luteum immunostained for von Willebrand factor, showing strong endothelial cell immunostaining in the vascular connective tissue invaginations (arrow); (**e**) dark-field view of a mid-luteal corpus luteum after isotopic in-situ hybridization for luteinizing hormone (LH) receptor mRNA, showing grains distributed over the steroidogenic cells; and (**f**) the same corpus luteum as (**e**) after immunohistochemistry for macrophages (CD68 positive cells) showing the distribution of macrophages around the periphery of the granulosa–lutein cell layer, a different localization from LH receptor mRNA. (G) Granulosa–lutein cell layer, (T) Theca–lutein cell layer (arrow) in (**a**) to (**d**) connective tissue invaginations. Scale bar = 100 μm.

supply. Endothelial cells were also scattered throughout the granulosa–lutein cell layers, particularly in the radial invaginations from the theca–lutein cell layer (Figure 1d). LH receptors were localized by isotopic mRNA in-situ hybridization (Figure 1e). As described previously, they were localized to the steroidogenic cell population (Duncan *et al.*, 1996b). When compared with immunostained sections, no hybridization signal could be detected in endothelial cells, stromal cells or cells lacking the morphological characteristics of steroidogenic cells.

Localization of tissue macrophages

Macrophages, as described by immunohistochemical localization of the CD68 antigen, could be localized in all corpora lutea studied (Figure 2a). No staining was present in negative control sections where the primary antibody was replaced with an equivalent concentration of IgG (Figure 2b). Many more macrophages could be detected in late-luteal corpora lutea (Figure 2c) than after luteal 'rescue' with exogenous HCG (Figure 2d). The

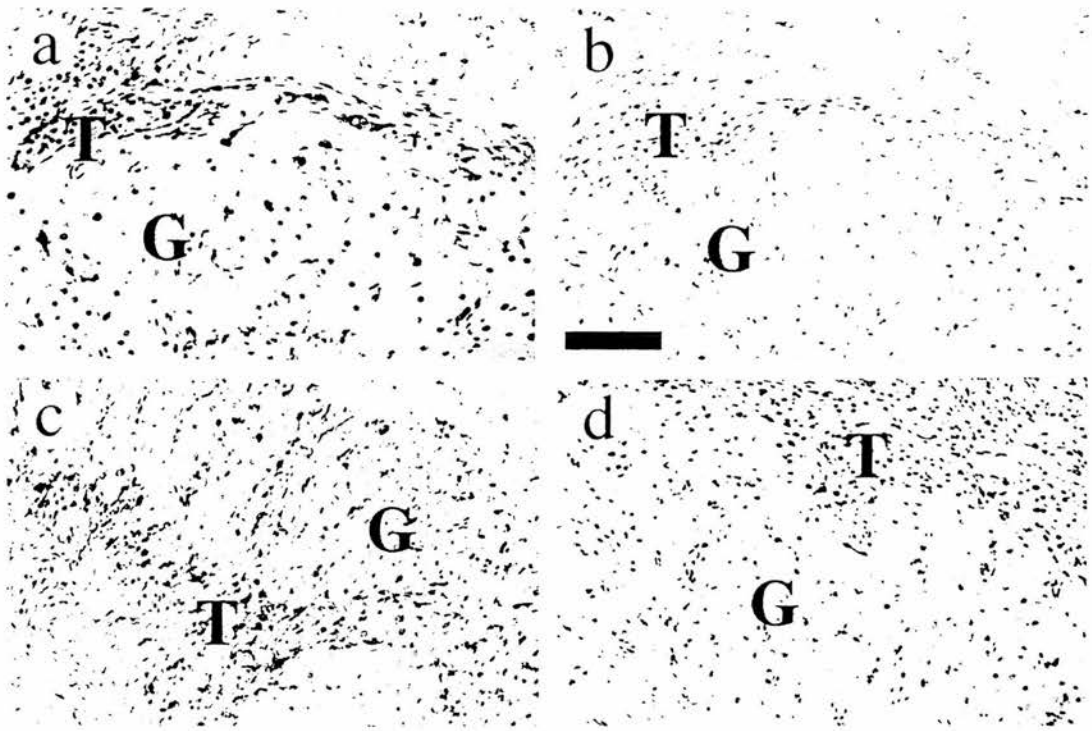

Figure 2. Macrophage localization in the human corpus luteum: (**a**) mid-luteal corpus luteum with macrophages (CD68 positive cells, brown) localized to the periphery of the steroidogenic cells in the theca–lutein layer (T) and also to the granulosa–lutein cell layer (G) and surrounding connective tissue stroma; (**b**) negative control serial section of (**a**) showing the theca–lutein (T) and granulosa–lutein (G) cell layers with no specific staining visible; (**c**) increased numbers of macrophages in the theca–lutein (T) and granulosa–lutein (G) cell layers in a late-luteal corpus luteum; and (**d**) corpus luteum after luteal 'rescue' with human chorionic gonadotrophin showing fewer macrophages in the theca–lutein (T) and granulosa–lutein (G) cell layers. Scale bar = 100 μm.

numbers of macrophages in the corpora lutea at different stages of the luteal phase were counted. The macrophage content of the corpus luteum increased throughout the luteal phase reaching a maximum in the late-luteal phase ($P < 0.05$) (Figure 3). Luteal 'rescue' with HCG was associated with a reduction in the number of macrophages (Figure 3), which was significantly lower than in the late-luteal phase ($P < 0.001$).

Macrophages were particularly prominent in the theca–lutein cell layer at all stages (Figure 1f, Figure 2a). In the granulosa–lutein cell layer, they were usually seen in association with the vascular in-foldings, particularly in the late-luteal phase (Figure 2c). The numbers of macrophages specifically located within the granulosa cell layer showed the same pattern as the overall macrophage content throughout the luteal phase (Figure 4). There were no differences in the percentages of macrophages specifically located to the granulosa–lutein cell layer at any stage of the luteal phase. The localization of the LH receptor (Figure 1e) in the human corpus luteum was clearly different from the localization of CD-68 positive tissue macrophages (Figure 1f).

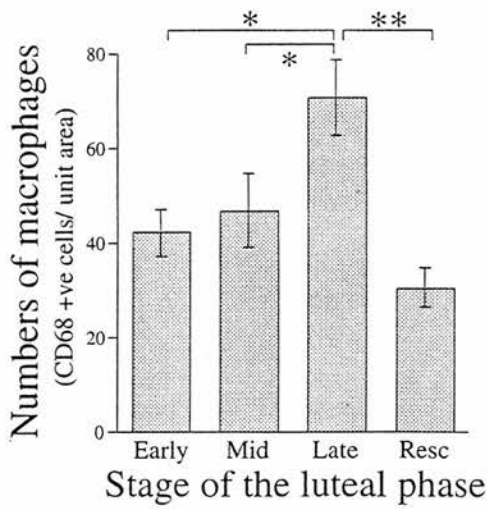

Figure 3. Macrophage numbers in corpora lutea: numbers of CD68 positive cells in sections of human corpus luteum in the early- LH+1 to LH+5, mid- (LH+6 to LH+10) and late- (LH+11 to LH+14) luteal phase and after luteal 'rescue' with human chorionic gonadotrophin (HCG) (HCG×5 to HCG×8). Values are mean ± SEM (n = 4 per group). Values were analysed by one-way analysis of variance and, where significant differences at the 5% level were observed, pairwise comparisons were conducted using the Bonferroni/Dunn method (*$P < 0.05$, **$P < 0.001$).

Discussion

We have compared the macrophage content in the corpora lutea of women who received exogenous HCG at concentrations equivalent to that of early pregnancy with that of corpora lutea obtained from untreated women at clearly defined stages of the luteal phase. We found that the macrophage content was significantly lower in the HCG-treated women than in the untreated women from the same stage of the luteal phase. This suggests that macrophage accumulation is associated with the loss of luteal integrity during luteolysis and that one of the

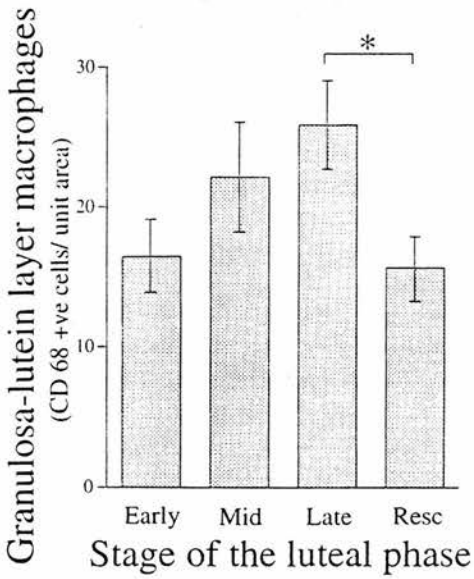

Figure 4. Macrophage numbers within the granulosa–lutein cell layer: numbers of CD68 positive cells in sections of human corpus luteum in the early-LH+1 to LH+5), mid- (LH+6 to LH+10) and late- (LH+11 to LH+14) luteal phase and after luteal 'rescue' with human chorionic gonadotrophin (HCG) (HCG×5 to HCG×8). Values are mean ± SEM (n = 4 per group). Values were analysed by one-way analysis of variance and, where significant differences at the 5% level were observed, pairwise comparisons were conducted using the Bonferroni/Dunn method ($^*P < 0.05$).

effects of HCG during luteal 'rescue' is to prevent the accumulation of macrophages in the corpus luteum.

In this study we have concentrated on the numbers and localization of macrophages in the corpus luteum. Various other immune cells have also been identified in the corpus luteum, including polymorphonuclear leukocytes (Brännström *et al.*, 1994b) and T-cells (Best *et al.*, 1996). However, it is clear that macrophages are the predominant immune cell subpopulation in the human and rabbit corpus luteum (Bagavandoss *et al.*, 1990; Wang *et al.*, 1992a; Best *et al.*, 1996). In addition, unlike other immune cells, macrophages have clearly been shown to vary in number during the lifespan of the corpus luteum, being particularly abundant in the regressing corpus luteum (Brännström *et al.*, 1994b; Best *et al.*, 1996). Although other immune cells may be affected during luteal 'rescue', it is likely that the clearest and most predominant effect is that on tissue macrophage content.

Our study confirms that macrophage influx increases during the functional lifespan of the corpus luteum. Macrophage accumulation in the corpus luteum is a feature of luteolysis in a variety of animal species, including rats (Brännström *et al.*, 1994a), rabbits (Bagavandoss *et al.*, 1990; Naftalin *et al.*, 1997), pigs (Hehnke *et al.*, 1994), sheep (Murdoch, 1987) and women (Brännström *et al.*, 1994b; Best *et al.*, 1996; Takaya *et al.*, 1997). Macrophages are clearly present in large numbers in the regressing corpus luteum after menstruation in women (Wang *et al.*, 1992a; Brännström *et al.*, 1994b; Takaya *et al.*, 1997). However, there has been some debate about the accumulation of macrophages in the late-luteal phase when the corpus luteum is still producing progesterone. Brännström *et al.* (1994b) failed to find an increase in luteal macrophages in the late-luteal phase. In contrast, other studies (Lei *et al.*, 1991; Best *et al.*, 1996) reported increased macrophages in the functional corpora lutea in the late-luteal phase. It is likely that the discrepant findings of Brännström *et al.* (1994b) are explained by their more extended definition of the late-luteal phase (from LH+8) and the fact that macrophage influx is a feature of luteal ageing.

The role of macrophage accumulation in the late-luteal phase is not fully established. It is not clear whether it is a cause or consequence of falling progesterone synthesis. Clearly macrophage products can inhibit steroidogenesis *in vitro*. Nitric oxide (Van Voorhis *et al.*, 1994), TNFα (Benyo and Pate, 1992; Wang *et al.*, 1992b), $PGF_{2\alpha}$ (Grusenmeyer and Pate, 1992), reactive oxygen species (Vega *et al.*, 1995; Kato *et al.*, 1997) and interleukins (Sjögren *et al.*, 1991) have all been shown to inhibit the steroidogenic pathway. In addition, cell death by apoptosis is a feature of luteolysis in many species (Dharmarajan *et al.*, 1994; Zheng *et al.*, 1994; Shikone *et al.*, 1996) and apoptosis can be promoted by macrophage products, such as free oxygen radicals, TNFα and some interleukins (Hale *et al.*, 1996; Jacobson, 1996; Spencer *et al.*, 1996). Macrophages may have a role in structural luteolysis; they can clear cellular debris by phagocytosis (Paavola, 1979) and activate the matrix metalloproteinase enzymes (Hurwitz *et al.*, 1993; Hulboy *et al.*, 1997) which have been implicated in the remodelling associated with luteolysis (Endo *et al.*, 1993; Luck and Zhao, 1995). Whether it is a cause or consequence of falling progesterone synthesis, it is likely that the accumulation of macrophages in the late-luteal phase has a negative effect on the structure and function of the corpus luteum.

Macrophages, however, have been shown to have both prosteroidogenic and luteotrophic properties under some conditions. It remains possible that the macrophage accumulation in the functional luteal phase is a luteotrophic response to failing progesterone synthesis. Macrophage-derived products have been shown to enhance progesterone output from luteal cells in culture (Kirsch *et al.*, 1983; Halme *et al.*, 1985), and macrophages may secrete factors important for angiogenesis and tissue reorganization. Brännström and Norman (1993) postulated a luteotrophic effect of macrophages in the early luteal phase. In addition, as macrophages could enhance progesterone synthesis (Kirsch *et al.*, 1981, 1983) and promote proliferation of granulosa cells (Fukumatsu *et al.*, 1992), Bukovsky *et al.* (1995) also proposed a luteotrophic role of macrophages. In the rat corpus luteum, non-steroidogenic cells, probably white blood cells, have also been shown to have potent stimulatory effects on luteal cell steroidogenesis (Nelson *et al.*, 1992). It is not clear, therefore, whether the accumulation of macrophages in the corpus luteum would always be associated with a fall in progesterone synthesis.

We have demonstrated that luteal 'rescue' with exogenous HCG to mimic the early stages of pregnancy is associated with a reduction in the numbers of macrophages in the late-luteal corpus luteum. This supports the hypothesis that macrophages have a primarily luteolytic, rather than luteotrophic, role in the human corpus luteum. Our findings are different from those of some sub-primate species. Brännström

et al. (1994a) found particularly high concentrations of macrophages in the rat corpus luteum during the early stages of pregnancy and pseudopregnancy. They found sixfold more macrophages in the corpus luteum of early pregnancy than during luteolysis (Brännström *et al.*, 1994a). This accumulation of macrophages is also seen in the corpus luteum of pregnant rabbits (Bagavandoss *et al.*, 1990). In the rabbit corpus luteum, oestrogen withdrawal induces macrophage invasion, but subsequent oestrogen replacement maintained progesterone production and did not necessarily reduce macrophage numbers (Naftalin *et al.*, 1997). Indeed, in that model system, the relative numbers of macrophages had no apparent relationship to progesterone synthesis. They concluded that the presence of macrophages did not preclude the continuation of progesterone production (Naftalin *et al.*, 1997). It is not known whether the human corpus luteum can continue to function in the presence of increasing numbers of macrophages but, clearly, macrophage influx is not a feature of luteal 'rescue' with HCG.

What promotes the influx of macrophages into the failing corpus luteum? Several chemoattractant and macrophage-stimulatory molecules have now been identified, including cytokines such as interleukin 8 (Norman and Brännström, 1994), granulocyte–macrophage colony stimulating factor (Nicola, 1989) and monocyte chemoattractant protein 1 (MCP-1) (Leonard and Yoshimura, 1990). These molecules can be detected in the ovary (Robertson and Seamark, 1990; Zhao *et al.*, 1995; Arici *et al.*, 1997). Recently, Townson *et al.* (1996) reported increased expression of MCP-1 in the corpus luteum during luteal regression in rats. This increase preceded the appearance of macrophages in the corpus luteum and they concluded that MCP-1 may have a prominent role in the immunological process of luteal regression. Induction of structural luteolysis by prolactin in rat corpus luteum (Bowen *et al.*, 1996), and by oestrogen withdrawal in the rabbit corpus luteum (Naftalin *et al.*, 1997), is associated with macrophage accumulation and expression of MCP-1. MCP-1 can be stimulated by cytokines (Oppenheim *et al.*, 1991; Arici *et al.*, 1997) which can be found in the corpus luteum during luteolysis (Brännström and Norman, 1993). However, MCP-1 expression can also be stimulated by HCG in granulosa–lutein cell culture (Arici *et al.*, 1997). Data on the expression of these chemoattractant molecules in the human corpus luteum throughout the luteal phase are not yet available, but would clearly be of great interest.

How does exposure to HCG during luteal 'rescue' prevent the influx of macrophages into the corpus luteum? HCG exerts its biological actions by binding to, and activating, LH receptors (Cole *et al.*, 1973). LH receptors are localized to the steroidogenic cells of the corpus luteum (Nishimori *et al.*, 1995; Duncan *et al.*, 1996b), and we have shown the localization of these receptors is different from the localization of macrophages. Although we were not able to co-localize macrophages and LH receptors on the same tissue section, it is unlikely that macrophages themselves express the LH receptor. The effect of HCG on macrophage accumulation therefore seems to be mediated through the steroidogenic cells. This effect is likely to be associated with the production or withdrawal of steroid or non-steroid molecules from the cells expressing LH/HCG receptors. Progesterone itself may function as a signal molecule as progesterone receptors can be localized to the human corpus luteum (Suzuki *et al.*, 1994). While these receptors appear to be present on steroidogenic cells (Suzuki *et al.*, 1994; Hild-Petito and Fazleabas, 1997), they can also be found on other cells within the connective tissue stroma (Suzuki *et al.*, 1994). It is not known whether luteal macrophages express progesterone receptors or whether luteal sex steroids can affect migration directly.

In our study, macrophages were found to be associated with the theca–lutein cell layer and the vasculature of the corpus luteum. An early study reported that macrophages were predominant in the granulosa–lutein cell layer of the human corpus luteum (Gillim *et al.*, 1969). However, later studies found a predominance in the theca–lutein layer (Wang *et al.*, 1992a, Brännström *et al.*, 1994b). We have used steroidogenic markers to confirm this observation. Brännström *et al.* (1994b) reported that macrophages were more abundant in the theca–lutein layer and were particularly associated with blood vessels. The relationship with blood vessels may suggest a recruitment of monocyte/macrophages from the circulation. In our study macrophage numbers increased during the late-luteal phase, in all cellular compartments. This is consistent with recruitment from the circulation rather than local migration. Interestingly, MCP-1 is secreted by several cell types including endothelial cells and fibroblasts (Leonard and Yoshimura, 1990). In the rat corpus luteum, luteal vascular cells appear to be a source of MCP-1 (Townson *et al.*, 1996). The endothelial cells and their communication with the steroidogenic cells of the corpus luteum may play a major role in the control of macrophage recruitment.

In conclusion, this study has shown that macrophages accumulate in the human corpus luteum during the luteal phase and are maximal in the late-luteal phase. One of the effects of HCG during luteal 'rescue' is to prevent this influx of macrophages into the corpus luteum. As macrophages do not express LH/HCG receptors, this effect is mediated indirectly through factors produced by the steroidogenic cells. Further work is needed to study the expression of chemoattractant molecules in the human corpus luteum throughout the luteal phase and after luteal 'rescue' with HCG.

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References

Arici, A., Oral, E., Bukulmez, O., Buradagunta, S. *et al.* (1997) Monocyte chemotactic protein-1 expression in human preovulatory follicles and ovarian cells. *J. Reprod. Immunol.*, **32**, 201–219.

Auletta, F.J. and Flint, A.P.F. (1988) Mechanisms controlling corpus luteum

function in sheep, cows, nonhuman primates, and women especially in relation to the time of luteolysis. *Endocrine Rev.*, **9**, 88–105.

Bagavandoss, P., Wiggins, R.C., Kunkel, S.L. *et al.* (1990) Tumor necrosis factor production and accumulation of inflammatory cells in the corpus luteum of pseudopregnancy and pregnancy in rabbits. *Biol. Reprod.*, **42**, 367–376.

Behrman, H.R., Endo, T., Aten, R.F. and Musicki, B. (1993) Corpus luteum function and regression. *Reprod. Med. Rev.*, **2**, 153–180.

Benyo, D.F. and Pate, J.L. (1992) Tumor necrosis factor-alpha alters bovine luteal cell synthetic capacity and viability. *Endocrinology*, **130**, 854–860.

Best, C.L., Pudney, J., Welch, W.R. *et al.* (1996) Localization and characterization of white blood cell populations within the human ovary throughout the menstrual cycle and menopause. *Hum. Reprod.*, **11**, 790–797.

Bowen, J.M., Keyes, P.L., Warren, J.S. and Townson, D.H. (1996) Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages. *Biol. Reprod.*, **54**, 1120–1127.

Brännström, M. and Norman, R.J. (1993) Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. *Hum. Reprod.*, **8**, 1762–1775.

Brännström, M., Giesecke, L., Moore, I.C. *et al.* (1994a) Leukocyte subpopulations in the rat corpus luteum during pregnancy and pseudopregnancy. *Biol. Reprod.*, **50**, 1161–1167.

Brännström, M., Pascoe, V., Norman, R.J. and McClure, N. (1994b) Localization of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. *Fertil. Steril.*, **61**, 488–495.

Bukovsky, A., Caudle, M.R., Keenan, J.A. *et al.* (1995) Quantitative evaluation of the cell cycle-related retinoblastoma protein and localization of Thy-1 differentiation protein and macrophages during follicular development and atresia and in human corpora lutea. *Biol. Reprod.*, **52**, 776–792.

Cole, F.E., Weed, J.C., Schneider, G.T. *et al.* (1973) The gonadotropin receptor of the human corpus luteum. *Am. J. Obstet. Gynecol.*, **117**, 87–95.

Dharmarajan, A.M., Goodman, S.B., Tilly, K.I. and Tilly, J.L. (1994) Apoptosis during functional corpus luteum regression: evidence of a role for chorionic gonadotropin in promoting luteal cell survival. *Endocrine J.*, **2**, 295–303.

Djahanbakhch, O., McNeilly, A.S., Hobson, B.M. and Templeton, A.A. (1981a) A rapid luteinizing hormone radioimmunoassay for the prediction of ovulation. *Br. J. Obstet. Gynaecol.*, **88**, 1016–1020.

Djahanbakhch, O., Swanston, I.A., Corrie, J.E.T. and McNeilly, A.S. (1981b) Prediction of ovulation by progesterone. *Lancet*, **ii**, 1164–1165.

Duncan, W.C., McNeilly, A.S. and Illingworth, P.J. (1996a) Expression of tissue inhibitor of metalloproteinases-1 in the human corpus luteum after luteal rescue. *J. Endocrinol.*, **148**, 59–67.

Duncan, W.C., McNeilly, A.S., Fraser, H.M. and Illingworth, P.J. (1996b) Luteinizing hormone receptor in the human corpus luteum: lack of down-regulation during maternal recognition of pregnancy. *Hum. Reprod.*, **11**, 2291–2297.

Endo, T., Aten, R.F., Wang, F. and Behrman, H.R. (1993) Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. *Endocrinology*, **133**, 690–698.

Fukumatsu, Y., Katabuchi, H., Naito, M. *et al.* (1992) Effect of macrophages on proliferation of granulosa cells in the ovary in rats. *J. Reprod. Fertil.*, **96**, 241–249.

Gillim, S.W., Christensen, A.K. and McLennan, C.E. (1969) Fine structure of the human menstrual corpus luteum at its stage of maximum secretory activity. *Am. J. Anat.*, **126**, 409–428.

Grusenmeyer, D.P. and Pate, J.L. (1992) Localization of prostaglandin $F_{2\alpha}$ inhibition of lipoprotein use by bovine luteal cells. *J. Reprod. Fertil.*, **94**, 311–318.

Hale, A.J., Smith, C.A., Sutherland, L.C. *et al.* (1996) Apoptosis: molecular regulation of cell death. *Eur. J. Biochem.*, **236**, 1–26.

Halme, J., Hammond, M.G., Syrop, C.H. and Talbert, L.M. (1985) Peritoneal macrophages modulate human granulosa-luteal cell progesterone production. *J. Clin. Endocrinol. Metab.*, **61**, 912–916.

Henke, K.E., Christenson, L.K., Ford, S.P. and Taylor, M. (1994) Macrophage infiltration into the porcine corpus luteum during prostaglandin $F_{2\alpha}$-induced luteolysis. *Biol. Reprod.*, **50**, 10–15.

Hild-Petito, S. and Fazleabas, A.T. (1997) Expression of steroid receptors and steroidogenic enzymes in the baboon (*Papio anubis*) corpus luteum during the menstrual cycle and early pregnancy. *J. Clin. Endocrinol. Metab.*, **82**, 955–962.

Hulboy, D.L., Rudolph, L.A. and Matrisian, L.M. (1997) Matrix metalloproteinases as mediators of reproductive function. *Mol. Hum. Reprod.*, **3**, 27–45.

Hurwitz, A., Dushnik, M., Solomon, H. *et al.* (1993) Cytokine-mediated regulation of rat ovarian function: interleukin-1 stimulates the accumulation of a 92-kilodalton gelatinase. *Endocrinology*, **132**, 2709–2714.

Illingworth, P.J., Reddi, K., Smith, K. and Baird, D.T. (1990) Pharmacologic 'rescue' of the corpus luteum results in increased inhibin production. *Clin. Endocrinol.*, **33**, 323–332.

Jacobson, M.D. (1996) Reactive oxygen species and programmed cell death. *Trends Biological Sci.*, **21**, 83–86.

Kato, H., Sugino, S., Takiguchi, S. *et al.* (1997) Roles of reactive oxygen species in the regulation of luteal function. *Rev. Reprod.*, **2**, 81–83.

Kirsch, T.M., Friedman, A.C., Vogel, R.L. and Flickinger, G.L. (1981) Macrophages in corpora lutea of mice: characterization and effects on steroid secretion. *Biol. Reprod.*, **25**, 629–638.

Kirsch, T.M., Vogel, R.L. and Flickinger, G.L. (1983) Macrophages: a source of luteotrophic cybernins. *Endocrinology*, **115**, 1910–1912.

Lei, Z.M., Chegini, N. and Rao, Ch.V. (1991) Quantitative cell composition of human and bovine corpora lutea from various reproductive states. *Biol. Reprod.*, **44**, 1148–1156.

Leonard, E.J. and Yoshimura, T. (1990) Human monocyte chemoattractant protein-1 (MCP-1). *Immunol. Today*, **11**, 97–101.

Li, T.C., Rogers, A.W., Dockery, P. *et al.* (1988) A new method of histologic dating of human endometrium in the luteal phase. *Fertil. Steril.*, **50**, 52–60.

Luck, M.R. and Zhao, Y. (1995) Structural remodelling of reproductive tissues. *J. Endocrinol.*, **146**, 191–195.

Murdoch, W.J. (1987) Treatment of sheep with prostaglandin F2 alpha enhances production of a luteal chemoattractant for eosinophils. *Am. J. Reprod. Immunol. Microbiol.*, **15**, 52–56.

Naftalin, D.M., Bove, S.E., Keyes, P.L. and Townson, D.H. (1997) Estrogen withdrawal induces macrophage invasion in the rabbit corpus luteum. *Biol. Reprod.*, **56**, 1175–1180.

Nelson, S.E., McLean, M.P., Jayatilak, P.G. and Gibori, G. (1992) Isolation, characterization, and culture of cell subpopulations forming the pregnant rat corpus luteum. *Endocrinology*, **130**, 954–966.

Nicola, N.A. (1989) Hemopoietic cell growth factors and their receptors. *Ann. Rev. Biochem.*, **58**, 45–77.

Nishimori, K., Dunkel, L., Hsueh, A.J.W. *et al.* (1995) Expression of luteinizing hormone and chorionic gonadotropin receptor messenger ribonucleic acid in human corpora lutea during menstrual cycle and pregnancy. *J. Clin. Endocrinol. Metab.*, **80**, 1444–1448.

Norman, R.J. and Brännström, M. (1994) White cells and the ovary - incidental invaders or essential effectors. *J. Endocrinol.*, **140**, 333–336.

Oppenheim, J.J., Zachariae, C.O.C., Mukaida, N. and Matsushima, K. (1991) Properties of the novel proinflammatory supergene 'intracrine' cytokine family. *Ann. Rev. Immunol.* **9**, 617–648.

Paavola, L.G. (1979) The corpus luteum of the guinea pig. IV. Fine structure of macrophages during pregnancy and postpartum luteolysis and the phagocytosis of luteal cells. *Am. J. Anat.*, **50**, 337–364.

Riley, S.C., Dupont, E., Walton, J.C. *et al.* (1992) Immunohistochemical localization of 3β-hydroxy-5-ene-steroid dehydrogenase/ Δ^5-Δ^4 isomerase in human placenta and fetal membranes throughout gestation. *J. Clin. Endocrinol. Metab.*, **75**, 956–961.

Robertson, S.A. and Seamark, R.F. (1990) Granulocyte macrophage colony stimulating factor (GM-CSF) in the murine reproductive tract: stimulation by seminal factors. *Reprod. Fertil. Dev.*, **2**, 359–368.

Rodger, F.E., Fraser, H.M., Duncan, W.C. and Illingworth, P.J. (1995) Immunolocalization of bcl-2 in the human corpus luteum. *Hum. Reprod.*, **10**, 1566–1570.

Rodger, F.E., Young, F.M., Fraser, H.M. and Illingworth, P.J. (1997) Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic gonadotrophin rescue, in the human corpus luteum. *Hum. Reprod.*, **12**, 1723–1729.

Shikone, T., Yamoto, M., Kokawa, K. *et al.* (1996) Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy. *J. Clin. Endocrinol. Metab.*, **81**, 2376–2380.

Sjögren, A., Holmes, P.V. and Hillensjo, T. (1991) Interleukin-1α modulates luteinizing hormone stimulated cyclic AMP and progesterone release from human granulosa cells *in vitro*. *Hum. Reprod.*, **6**, 910–913.

Spencer, S.J., Cataldo, N.A. and Jaffe, R.B. (1996) Apoptosis in the human female reproductive tract. *Obstet. Gynecol. Survey*, **51**, 314–323.

Suzuki, T., Sasano, H., Kimura, N. *et al.* (1994) Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: relationship to expression of steroidogenic enzymes. *Hum. Reprod.*, **9**, 1589–1595.

Takaya, R., Fukaya, T., Sasano, H. *et al.* (1997) Macrophages in normal

cycling human ovaries; immunohistochemical localization and characterization. *Hum. Reprod.*, **12**, 1508–1512.

Townson, D.H., Warren, J.S., Flory, C.M. *et al.* (1996) Expression of Monocyte Chemoattractant Protein-1 in the corpus luteum of the rat. *Biol. Reprod.*, **54**, 513–520.

Van Voorhis, B.J., Dunn, M.S., Snyder, G.D. and Weiner, C.P. (1994) Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology*, **135**, 1799–1805.

Vega, M., Carrasco, I., Castillo, T. *et al.* (1995) Functional luteolysis in response to hydrogen peroxide in human luteal cells. *J. Endocrinol.*, **147**, 177–182.

Wang, H.Z., Pascoe, V., Petrucco, O.M. and Norman, R.J. (1992a) Distribution of leukocyte subpopulations in the human corpus luteum. *Hum. Reprod.*, **7**, 197–202.

Wang, H.Z., Lu, S.H., Han, X.J. *et al.* (1992b) Inhibitory effect of interferon and tumor necrosis factor on human luteal function *in vitro*. *Fertil. Steril.*, **58**, 941–945.

Zhao, Y., Rong, H. and Chegini, N. (1995) Expression and selective cellular localization of granulocyte-macrophage colony-stimulating factor (GM-CSF) and GM-CSF α and β receptor messenger ribonucleic acid and protein in human ovarian tissue. *Biol. Reprod.*, **53**, 923–930.

Zheng, J., Fricke, P.M., Reynolds, L.P. and Redmer, D.A. (1994) Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. *Biol. Reprod.*, **51**, 623–632.

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